

---

# Biotechnology in Agriculture and Forestry

Edited by T. Nagata  
H. Lörz and J.M. Widholm

---

## **60** Transgenic Crops V

Edited by E.C.Pua and M.R. Davey

# Biotechnology in Agriculture and Forestry

---

Edited by

T. Nagata (Managing Editor)

H. Lörz

J. M. Widholm

# Biotechnology in Agriculture and Forestry

---

Volumes already published and in preparation are listed at the end of this book.

---

# Biotechnology in Agriculture and Forestry 60

---

## *Transgenic Crops V*

Edited by  
E.C. Pua and M.R. Davey

With 11 Figures and 33 Tables



Springer

*Series Editors*

Professor Dr. TOSHIYUKI NAGATA  
University of Tokyo  
Graduate School of Science  
Department of Biological Sciences  
7-3-1 Hongo, Bunkyo-ku  
Tokyo 113-0033, Japan

Professor Dr. HORST LÖRZ  
Universität Hamburg  
Institut für Allgemeine Botanik  
Angewandte Molekularbiologie  
der Pflanzen II  
Ohnhorststraße 18  
22609 Hamburg, Germany

Professor Dr. JACK M. WIDHOLM  
University of Illinois  
285A E.R. Madigan Laboratory  
Department of Crop Sciences  
1201 W. Gregory  
Urbana, IL 61801, USA

*Volume Editors*

Professor Dr. ENG-CHONG PUA  
School of Arts and Sciences  
Monash University Malaysia  
2 Jalan Kolej, Bandar Sunway  
46150 Petaling Jaya, Selangor, Malaysia

Professor Dr. MICHAEL R. DAVEY  
Plant Sciences Division  
School of Biosciences  
University of Nottingham  
Sutton Bonington Campus  
Loughborough LE12 5RD, UK

Library of Congress Control Number: 2006933056

ISSN 0934-943X

ISBN-13 978-3-540-49160-6 Springer Berlin Heidelberg New York

This work is subject to copyright. All rights reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilm or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permission for use must always be obtained from Springer. Violations are liable for prosecution under the German Copyright Law.

Springer is a part of Springer Science + Business Media  
springer.com

© Springer-Verlag Berlin Heidelberg 2007

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Editor: Dr. Dieter Czeschlik, Heidelberg, Germany  
Desk Editor: Dr. Andrea Schlitzberger, Heidelberg, Germany  
Cover design: WMXdesign, Heidelberg, Germany  
Typesetting and production: LE-TeX Jelonek, Schmidt & Vöckler GbR, Leipzig, Germany  
Printed on acid-free paper SPIN 10981542 31/3100 5 4 3 2 1 0

*Dedicated by E.C. Pua  
to his wife (Pat-Mui Fong)  
and children (Seow Hun, Seow Chin and Xuan Zhan)*

## Preface

Exciting developments in crop biotechnology in recent years have prompted the necessity to update the first series of *Transgenic Crops I, II* and *III*, published in 1999 and 2001. In this current endeavor, 69 chapters have been compiled, contributed by a panel of experts in crop biotechnology from 26 countries. These chapters are grouped into three volumes, namely *Transgenic Crops IV*, *V* and *VI*. This new series not only reviews recent advances in cell and tissue culture and genetic transformation methodologies, but also presents aspects of the molecular genetics of target crops and the practical applications of transgenic plants. In addition, more than 30% of crop species that were not discussed previously are included in the present series.

This new series commences with the volume *Transgenic Crops IV*, consisting of 23 chapters that focus on cereals, vegetables, root crops, herbs and spices. Section I is an introductory chapter that places into perspective the impact of plant biotechnology in agriculture. Section II focuses on cereals (rice, wheat, maize, rye, pearl millet, barley, oats), while Section III is directed to vegetable crops (tomato, cucumber, eggplant, lettuce, chickpea, common beans and cowpeas, carrot, radish). Root crops (potato, cassava, sweet potato, sugar beet) are included in Section IV, with herbs and spices (sweet and hot peppers, onion, garlic and related species, mint) in Section V.

*Transgenic Crops V* also consists of 23 chapters in three sections devoted to fruit (Section I), trees (Section II) and beverage crops (Section III). Fruit crops target banana, citrus, mango, papaya, pineapple, watermelon, avocado, grape, melon, apple, *Prunus* spp, strawberry and kiwifruit, while trees include rubber, eucalyptus, legumes and conifers. Section III, on beverage crops, reports studies on coffee, cacao, tea and sugarcane.

As in volumes IV and V, *Transgenic Crops VI* has 23 chapters organized in five Sections. Section I targets oil and fiber crops (soybean, rapeseed, sunflower, oil palm, peanut, cotton, flax), followed by medicinally important plants (including ginseng, opium poppy, herbane, belladonna, *Datura*, *Duboisia*, *Taxus*) in Section II. Ornamentals (roses, carnation, chrysanthemum, orchids, gladiolus, forsythia) are discussed in Section III, while Section IV involves forages and grains (alfalfa, clovers, tall fescue, ryegrasses, lupin). Section V has one chapter that discusses aspects of the freedom to commercialize transgenic plants, together with regulatory and intellectual property issues.

The editors express their sincere thanks to Maggie Yap Lan from Monash University, Malaysia, for her excellent secretarial and editorial assistance. She

forwarded to contributors timely reminders of deadlines, where appropriate, and assisted in editing the manuscripts for typographical errors and formatting.

This series will serve as a key reference for advanced students and researchers in crop sciences, genetics, horticulture, agronomy, cell and molecular biology, biotechnology and other disciplines in life sciences.

E.C. Pua and M.R. Davey



# Contents

## Section I Fruits

I.1	Banana . . . . .	3
	E.C. PUA	
1	Introduction . . . . .	3
2	Banana Breeding and Molecular Markers . . . . .	4
3	Identification of Banana Genes . . . . .	5
4	Tissue Culture . . . . .	12
5	Genetic Transformation . . . . .	21
6	Factors Affecting Transgene Expression . . . . .	23
7	Applications of Transgenic Plants . . . . .	25
8	Conclusions . . . . .	26
	References . . . . .	26
I.2	Citrus . . . . .	35
	L. PEÑA, M. CERVERA, C. FAGOAGA, J. ROMERO, J. JUÁREZ, J.A. PINA, and L. NAVARRO	
1	Introduction . . . . .	35
2	The Importance of Citrus . . . . .	35
3	Genetic Improvement . . . . .	36
4	Tissue Culture . . . . .	37
5	Genetic Transformation . . . . .	38
6	Citrus Improvement by Genetic Engineering . . . . .	42
7	Conclusions . . . . .	47
	References . . . . .	47
I.3	Mango . . . . .	51
	M.A. GÓMEZ LIM and R.E. LITZ	
1	Introduction . . . . .	51
2	Cell and Tissue Culture . . . . .	52
3	Applications of Biotechnology . . . . .	58
4	Conclusions . . . . .	66
	References . . . . .	67

I.4	Papaya . . . . .	73
	S.D. YEH	
1	Introduction . . . . .	73
2	Worldwide Threat by PRSV Infection . . . . .	75
3	A Transgenic Approach for Control of PRSV . . . . .	76
4	Tissue Culture and Regeneration Techniques . . . . .	79
5	Micropropagation Techniques . . . . .	81
6	Methods of Genetic Transformation . . . . .	84
7	Advances in Molecular Biology . . . . .	87
8	Conclusions . . . . .	89
	References . . . . .	90
I.5	Pineapple . . . . .	97
	M.R. DAVEY, S. SRIPAORAYA, P. ANTHONY, K.C. LOWE, AND J.B. POWER	
1	Introduction . . . . .	97
2	Taxonomy and Breeding of Pineapple . . . . .	98
3	Genetic Relationships in Pineapple: Application of Biochemical and Molecular Markers . . . . .	99
4	Biochemical and Molecular Approaches to Flowering and Fruit Production in Pineapple . . . . .	100
5	Tissue Culture-Based Technologies for Pineapple Improvement . . .	102
6	Agronomic Improvement of Pineapple Using Genetic Manipulation: The Options . . . . .	111
7	Conclusions . . . . .	119
	References . . . . .	121
I.6	Watermelon . . . . .	129
	P. ELLUL, C. LELIVELT, M.M. NAVAL, F.J. NOGUERA, S. SANCHEZ, A. ATARÉS, V. MORENO, P. CORELLA, and R. DIRKS	
1	Introduction . . . . .	129
2	Watermelon Origin and Diversity . . . . .	129
3	Watermelon Cultivars and Germplasm . . . . .	131
4	Breeding Watermelon for Chromosome Doubling . . . . .	132
5	Genetic Engineering and Watermelon Breeding Purposes . . . . .	140
6	Production of Haploid and Doubled Haploid Watermelon Plants . .	150
7	DNA Markers in Watermelon Breeding . . . . .	152
8	Conclusions and Future Prospects . . . . .	155
	References . . . . .	156
I.7	Avocado . . . . .	167
	R.E. LITZ, S.H.T. RAHARJO, and M.A. GÓMEZ LIM	
1	Introduction . . . . .	167
2	Breeding and Genetics . . . . .	168

3	Molecular Genetics .....	170
4	Micropropagation .....	173
5	Micrografting for Elimination of Pathogens .....	176
6	Somatic Embryogenesis .....	177
7	Protoplast Isolation and Culture .....	178
8	In Vitro Mutation Induction .....	179
9	Genetic Transformation .....	180
10	Cryopreservation .....	181
11	Conclusions .....	182
	References .....	182
I.8	Grape .....	189
	A. PERL and Y. ESHDAT	
1	Introduction .....	189
2	Somatic Embryogenesis in Grapes .....	190
3	Selection Systems Utilizing Grape Cell Cultures .....	191
4	The International Grape Genome Program .....	192
5	Genetic Transformation in Viticulture .....	193
6	Utilization of Transgenic Grapes .....	193
7	Recent Advances in Grape Molecular Biology .....	198
8	Conclusions .....	202
	References .....	202
I.9	Melon .....	209
	J.C. PECH	
1	Introduction .....	209
2	Molecular Tools, Markers and Mapping .....	211
3	Molecular Characterization of Resistance Genes .....	216
4	In Vitro Regeneration by Organogenesis and Somatic Embryogenesis from Callus and Protoplasts .....	218
5	Haploidization, Triploids and Somatic Hybridisation .....	222
6	Methods of Genetic Transformation .....	222
7	Genetic Transformation for Disease Resistance .....	225
8	Genetic Transformation for Desirable Fruit Quality Traits and Post-Harvest Behaviour .....	226
9	Genetic Transformation for Resistance to Salt and Herbicides .....	229
10	Conclusions .....	230
	References .....	231
I.10	Apple .....	241
	A. M. IBANEZ and A. M. DANDEKAR	
1	Introduction .....	241
2	Economic Importance .....	244
3	Current Research and Development .....	245

4	Practical Applications of Transgenic Plants . . . . .	263
5	Conclusions and Future Challenges . . . . .	267
	References . . . . .	267
I.11	<i>Prunus</i> spp. . . . .	283
	L. BURGOS, C. PETRI, and M.L. BADENES	
1	Introduction . . . . .	283
2	Economic Importance . . . . .	283
3	Current Research and Development . . . . .	284
4	Practical Applications of Transgenic Plants . . . . .	296
5	Conclusions and Future Challenges . . . . .	296
	References . . . . .	300
I.12	Strawberry . . . . .	309
	J.A. MERCADO, F. PLIEGO-ALFARO, and M.A. QUESADA	
1	Introduction . . . . .	309
2	Economic Importance . . . . .	310
3	Current Research and Development . . . . .	311
4	Practical Applications of Transgenic Plants . . . . .	319
5	Conclusions and Future Challenges . . . . .	323
	References . . . . .	323
I.13	Kiwifruit . . . . .	329
	R.G. ATKINSON and E.A. MACRAE	
1	Introduction . . . . .	329
2	Economic Importance and Genetic Potential . . . . .	330
3	Molecular Biology . . . . .	334
4	Genetic Transformation and Functional Genomics . . . . .	337
5	Current and Future Practical Applications of Biotechnology . . . . .	340
	References . . . . .	341

## Section II Trees

II.1	Walnuts . . . . .	349
	M.T. BRITTON, C.H. LESLIE, G.H. McGRANAHAN, and A.M. DANDEKAR	
1	Introduction . . . . .	349
2	Economic Importance . . . . .	352
3	Current Research and Development . . . . .	353
4	Practical Applications of Transgenic Plants . . . . .	361
5	Conclusions and Future Challenges . . . . .	363
	References . . . . .	364

II.2	Rubber . . . . .	371
	P. AROKIJARAJ	
1	Introduction . . . . .	371
2	The Malaysian Rubber Industry . . . . .	371
3	<i>Hevea</i> Tissue Culture and Genetic Transformation . . . . .	372
4	Rubber Trees as Recombinant Protein Factories . . . . .	374
5	Molecular Genetics for <i>Hevea</i> Improvement . . . . .	378
6	Biosafety Aspects of Transgenic <i>Hevea</i> . . . . .	380
7	Conclusions and Future Challenges . . . . .	381
	References . . . . .	382
II.3	Eucalyptus . . . . .	387
	C. TEULIERES and C. MARQUE	
1	The Importance of Eucalyptus . . . . .	387
2	Tissue Culture and Genetic Transformation . . . . .	389
3	Eucalyptus Genomics Research Programs . . . . .	394
4	Conclusions and Perspectives . . . . .	401
	References . . . . .	402
II.4	Tropical Tree Legumes . . . . .	407
	Y. HONG and S. BHATNAGAR	
1	Introduction . . . . .	407
2	Progress and Prospects of Tree Legume Biotechnology . . . . .	408
3	In Vitro Propagation of Legume Tree Species . . . . .	408
4	Genetic Transformation . . . . .	419
5	Marker Assisted Breeding . . . . .	423
6	Genomic and Molecular Biology Studies . . . . .	424
7	Legume Nodulation and Nitrogen Fixation . . . . .	425
8	<i>Acacia mangium</i> as a Potential Model Plant for Tree Legumes . . . .	426
9	Conclusions . . . . .	426
	References . . . . .	427
II.5	Casuarina glauca . . . . .	433
	M. OBERTELLO, C. SANTI, S. SVISTOONOFF, V. HOCHER, F. AUGUY, L. LAPLAZE, D. BOGUSZ, and C. FRANCHE	
1	Introduction . . . . .	433
2	Economic Importance . . . . .	433
3	Transgenic Technology . . . . .	434
4	Transgenic Plants Used to Characterize Early Symbiotic Genes . . . .	435
5	Transgenic Casuarinaceae as a Tool for Evolutionary Studies of Symbiotic Genes . . . . .	437
6	Gene Transfer Technology for the Improvement of <i>C. glauca</i> . . . .	439
7	Conclusions . . . . .	442
	References . . . . .	442

II.6	Conifers .....	447
	C. WALTER, M. CARSON, and S. CARSON	
1	Introduction .....	447
2	The Economic Importance of Conifer Plantations .....	448
3	State of Current Research, Including Tissue Culture, Propagation, Molecular Genetics and Genetic Engineering .....	451
4	Potential Applications for Transgenic Conifers in Plantation Forestry .....	456
5	Social Challenges .....	459
6	Conclusions .....	466
	References .....	466

### Section III Beverage Crops

III.1	Coffee .....	475
	N. SANTANA, R. ROJAS-HERRERA, R.M. GALAZ-ÁVALOS, J.R. KU-CAUICH, J. MIJANGOS, and V.M. LOYOLA-VARGAS	
1	Introduction .....	475
2	Somatic Embryogenesis in Coffee .....	475
3	Other Tissue Culture Methods .....	482
4	Micropropagation .....	485
5	Genetic Transformation .....	486
6	Conclusions .....	489
	References .....	490
III.2	Cacao .....	497
	M.J. GUILTINAN	
1	Introduction .....	497
2	Recent Advances: A Recent Literature Review .....	500
3	Future Prospects .....	511
	References .....	513
III.3	Tea .....	519
	T.K. MONDAL	
1	Introduction .....	519
2	The Genome .....	520
3	Tea Breeding .....	520
4	Tissue Culture .....	521
5	Genetic Transformation .....	524
6	Molecular Genetics .....	525
7	Conclusions .....	530
	References .....	531

III.4	Sugarcane . . . . .	537
	L.H. ZHANG, L.X. WENG, and Z.D. JIANG	
1	Introduction . . . . .	537
2	Sugarcane Genetic Transformation . . . . .	540
3	Genetic Improvement . . . . .	541
4	Factors Affecting Transgene Expression and Performance . . . . .	546
5	Conclusions and Perspectives . . . . .	548
	References . . . . .	549
	Subject Index . . . . .	553

## List of Contributors

P. ANTHONY

Plant Sciences Division, School of Biosciences, University of Nottingham,  
Sutton Bonington Campus, Loughborough LE12 5RD, UK

P. AROKIARAJ

International Islamic University Malaysia, Kulliyyah of Science, Department  
of Biotechnology, Jalan Istana Bandar, Indra Mahkota, 25200 Kuantan,  
Pahang, Malaysia, e-mail: arokiaraj@iiu.edu.my

A. ATARÉS

IBMCP (Instituto De Biología Molecular Y Celular De Plantas), Avenida De  
Los Naranjos S/N, 46022 Valencia, Spain

R.G. ATKINSON

The Horticulture and Food Research Institute of New Zealand Ltd., Mt Albert  
Research Centre, Private Bag 92169, Auckland, New Zealand,  
e-mail: ross.atkinson@hortresearch.co.nz

F. AUGUY

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche  
pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394  
Montpellier Cedex 5, France

M.L. BADENES

Departamento de Citricultura y Otros Frutales, IVIA, Apartado de Correos,  
Moncada, 46.113 Valencia, Spain

H.J. BAU

Department of Biotechnology, Transworld Institute of Technology, Yunlin,  
Taiwan

A. BERNADAC

INRA/INPT-ENSAT “Génomique et Biotechnologie des Fruits” (UMR 990),  
Av. de l’Agrobiopole, BP 32607, 31326 Castanet-Tolosan Cedex, France



S. BHATNAGAR

Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore

D. BOGUSZ

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France

M. BOUZAYEN

INRA/INPT-ENSAT "Génomique et Biotechnologie des Fruits" (UMR 990), Av. de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan Cedex, France

M.T. BRITTON

Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA, e-mail: amdandekar@ucdavis.edu

L. BURGOS

Departamento de Mejora de Frutales, CEBAS-CSIC, Apartado de Correos 164, 30.100 Murcia, Spain, e-mail: burgos@cebas.csic.es

M. CARSON

Carson Associates Ltd, 34 Parawai Rd, Rotorua 3202, New Zealand

S. CARSON

Carson Associates Ltd, 34 Parawai Rd, Rotorua 3202, New Zealand

M. CERVERA

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

P. CORELLA

Rijk Zwaan Ibérica, Paraje El Mamí, Carretera De Viator S/N, 04120 La Cañada (Almería), Spain

A.M. DANDEKAR

Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA, e-mail: amdandekar@ucdavis.edu

M.R. DAVEY

Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK, e-mail: mike.davey@nottingham.ac.uk

R. DIRKS

Rijk Zwaan Breeding BV, 1e Kruisweg 9, 4793 RS Fijnaart, The Netherlands

C. DOGIMONT

INRA, Unité de Génétique et Amélioration des Fruits et Légumes, BP 94, 84143 Avignon Cedex, France

P. ELLUL

Rijk Zwaan Ibérica, Paraje El Mamí, Carretera De Viator S/N, 04120 La Cañada (Almería), Spain, e-mail: p.ellul@rijkszwaan.es

Y. ESHDAT

Department of Fruit Tree Sciences, Institute of Plant Science, Agricultural Research Organization, P.O. Box 6, 50250 Bet-Dagan, Israel

C. FAGOAGA

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

C. FRANCHE

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France, e-mail: franche@mpl.ird.fr

M.A. GÓMEZ LIM

CINVESTAV, Unidad Irapuato, Apartado Postal 629, Irapuato GTO 36500, Mexico, e-mail: mgomez@ciea.ira.cinvestav.mx

R.M. GALAZ-ÁVALOS

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, México

M.J. GUILTINAN

Department of Horticulture, 422 Life Sciences Building, The Pennsylvania State University, University Park, Pennsylvania 16802, USA, e-mail: mjpg9@psu.edu

V. HOCHER

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France

Y. HONG

Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore, e-mail: hongy@tll.org.sg

A.M. IBANEZ

Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA

Z.D. JIANG

Department of Plant Pathology, South China Agricultural University, Guangzhou 510642, China

J. JUÁREZ

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

J.R. KU-CAUICH

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, México

Y.J. KUNG

Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan

L. LAPLAZE

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France

A. LATCHE

INRA/INPT-ENSAT "Génomique et Biotechnologie des Fruits" (UMR 990), Av. de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan Cedex, France

C. LELIVELT

Rijk Zwaan Breeding BV, 1e Kruisweg 9, 4793 RS Fijnaart, The Netherlands

C.H. LESLIE

Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA

R.E. LITZ

Tropical Research and Education Center, University of Florida, 18905 SW 280 St, Homestead, Florida 33031-3314, USA, e-mail: rel@ifas.ufl.edu

K.C. LOWE

School of Biology, University of Nottingham, University Park, Nottingham  
NG7 2RD, UK

V.M. LOYOLA-VARGAS

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de  
Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de  
Hidalgo, Mérida, Yucatán, México, e-mail: vmloyola@cicy.mx

E.A. MACRAE

The Horticulture and Food Research Institute of New Zealand Ltd., Mt Albert  
Research Centre, Private Bag 92169, Auckland, New Zealand

C. MARQUE

Pôle de Biotechnologie Végétale, 24, Chemin de Borde Rouge, Auzeville,  
31326 Castanet Tolosan, France

G.H. MCGRANAHAN

Department of Plant Sciences, University of California, 1 Shields Ave, Davis,  
California 95616, USA

J.A. MERCADO

Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga,  
Spain, e-mail: mercado@uma.es

J. MIJANGOS

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de  
Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de  
Hidalgo, Mérida, Yucatán, México

T.K. MONDAL

Department of Plantation Crops and Processing, Uttar Banga Krishi  
Viswavidyalaya, Pundibari, Cooch Behar, 736165, West Bengal, India,  
e-mail: mondaltk@yahoo.com

V. MORENO

IBMCP (Instituto De Biología Molecular Y Celular De Plantas), Avenida De  
Los Naranjos S/N, 46022 Valencia, Spain

M.M. NAVAL

IVIA (Instituto Valenciano De Investigaciones Agrarias), Carretera Moncada  
– Náquera, Km. 4,5 Apartado Oficial, 46113 Moncada (Valencia), Spain

L. NAVARRO

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

F.J. NOGUERA

Rijk Zwaan Ibérica, Paraje El Mamí, Carretera De Viator S/N, 04120 La Cañada (Almería), Spain

M. OBERTELLO

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France

L. PEÑA

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain, e-mail: lpenya@ivia.es

J.C. PECH

INRA/INPT-ENSAT "Génomique et Biotechnologie des Fruits" (UMR 990), Av. de l'Agrobiopole, BP 32607, 31326 Castanet-Tolosan Cedex, France, e-mail: pech@ensat.fr

A. PERL

Department of Fruit Tree Sciences, Institute of Plant Science, Agricultural Research Organization, P.O. Box 6, 50250 Bet-Dagan, Israel, e-mail: perl@int.gov.il

C. PETRI

Departamento de Mejora de Frutales, CEBAS-CSIC, Apartado de Correos 164, 30.100 Murcia, Spain

J.A. PINA

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

M. PITRAT

INRA, Unité de Génétique et Amélioration des Fruits et Légumes, BP 94, 84143 Avignon Cedex, France

F. PLIEGO-ALFARO

Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga, Spain

J.B. POWER

Plant Sciences Division, School of Biosciences, University of Nottingham,  
Sutton Bonington Campus, Loughborough LE12 5RD, UK

E.C. PUA

School of Arts and Sciences, Monash University Malaysia, No. 2, Jalan  
Universiti, Bandar Sunway, 46150 Petaling Jaya, Selangor, Malaysia,  
e-mail: [ecpua@monash.edu.my](mailto:ecpua@monash.edu.my)

M.A. QUESADA

Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga,  
Spain

S.H.T. RAHARJO

Tropical Research and Education Center, University of Florida, 18905 SW 280  
St, Homestead, Florida 33031-3314, USA

R. ROJAS-HERRERA

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de  
Jalisco, Av. Normalistas 800, Guadalajara, Jalisco, México

J. ROMERO

Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de  
Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia,  
Spain

S. SANCHEZ

Rijk Zwaan Ibérica, Paraje El Mamí, Carretera De Viator S/N, 04120 La  
Cañada (Almería), Spain

N. SANTANA

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de  
Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de  
Hidalgo, Mérida, Yucatán, México

C. SANTI

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche  
pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394  
Montpellier Cedex 5, France

S. SRIPAORAYA

Faculty of Agriculture Nakhonsithammarat, Rajamangala University of  
Technology Srivijaya, Tungyai, Nakhonsithammarat 80240, Thailand

S. SVISTOONOFF

Laboratoire Rhizogenèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France

C. TEULIERES

Pôle de Biotechnologie Végétale, 24, Chemin de Borde Rouge, Auzeville, 31326 Castanet Tolosan, France, e-mail: teulieres@scsv.ups-tlse.fr

C. WALTER

Cellwall Biotechnology Center, SCION – Next Generation Biomaterials, Te Papa Tipu Innovation Park, 49 Sala Street, Private Bag 3020, Rotorua, New Zealand, e-mail: christian.walter@scionresearch.com

L.X. WENG

Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673

S.D. YEH

Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan, e-mail: sdyeh@nchu.edu.tw

T.A. YU

Department of Biotechnology, Da-Yeh University, Changhua, Taiwan

L.H. ZHANG

Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, e-mail: lianhui@imcb.a-star.edu.sg

## Section I Fruits



## I.1 Banana

E.C. PUA<sup>1</sup>

### 1 Introduction

Bananas (*Musa* spp.), belonging to the family Musaceae, are the perennial monocotyledons commonly grown in the tropics situated at latitude 20° above and below the equator, where there is a wide seasonal variation in rainfall and temperature. According to the FAO in 2004, 98% of world banana production was derived from developing countries, amongst which India, Brazil, China and Ecuador accounted for 50% of the total production (<http://faostat.fao.org/default.aspx>). It has been estimated that world production of bananas in 2004 was 70.6 million tons and the export value amounted to more than US \$4 billion annually. In international trade, bananas account for ~22% of world fresh fruit production and are ranked as the second most important fruit crop after citrus. Bananas can also be socio-politically important as they are the staple food of millions of people in the developing world.

Bananas comprise both wild and cultivated genotypes with considerable genetic diversity and a wide range of morphological characteristics. In general, bananas can be classified into four genome groups – A, B, S and T – represented by *M. acuminata* ( $2n = 2x = 22$ ), *M. balbisiana* ( $2n = 2x = 22$ ), *M. schizocarpa* ( $2n = 2x = 22$ ) and *Australimusa* ( $2n = 2x = 20$ ), respectively. The haploid genome size of bananas is relatively small at 500–600 Mbp (Lysák et al. 1999). Analysis of gene content and density in the genome of *M. acuminata* reveals that it is similar to the Graminae, as genes are usually clustered in the gene-rich regions, separated by the gene-poor domains that are rich in transposons (Aert et al. 2004).

Most commercial bananas are triploids ( $2n = 3x = 33$ ), with the genome of AAA, AAB and ABB originating from polyploidization and interspecific hybridization of the two diploid species *M. acuminata* and *M. balbisiana*. However, not all triploids are important commercially. Most cultivars involved in international trade belong to the Cavendish sub-group (*Musa* AAA), although they account for only 12% of total banana production (Robinson 1995).

---

<sup>1</sup> School of Arts and Sciences, Monash University Malaysia, No. 2, Jalan Universiti, Bandar Sunway, 46150 Petaling Jaya, Selangor, Malaysia, e-mail: [ecpua@monash.edu.my](mailto:ecpua@monash.edu.my)

## 2 Banana Breeding and Molecular Markers

Major efforts in different countries, including Australia, India, Brazil, Honduras and France, have been devoted to improving banana performance through breeding programs. The FAO/IAEA have supported a mutation-assisted breeding program for banana since 1994 (Jain 2005). Breeders aim to generate new banana hybrids with improved resistance to *Fusarium* wilt and Sigatoka disease, increased tolerance to nematodes and physical stress of the fruit, dwarfism, increased fruit yield and improved fruit flavour and morphology (Ortiz et al. 1995). In general, the progress of banana breeding has been relatively slow. This is due mainly to the narrow genetic variability resulting from the limited number of landraces selected from the natural germplasm, and low female fertility. Mutation treatment has been employed by subjecting cultured tissues to gamma irradiation to increase genetic variability and maintain fertility. This approach has resulted in the production of aluminium-tolerant (Matsumoto and Yamaguchi 1990) and early-flowering (Novak et al. 1990) variants. However, there has been limited success in producing novel banana variants with commercial importance by induced mutation.

As part of the breeding programs, banana research has focused on genome structure analysis and the development of biochemical and molecular markers. It has been reported that genomic in situ hybridization can be used to differentiate the chromosomes of four genome groups and interspecific cultivars of banana (D'Hont et al. 2000), while the distribution of repeated sequences in the genome can be determined by fluorescence in situ hybridization (D'Hont 2005). These techniques are useful for the development of DNA markers, as reviewed by Jarret and Gawel (1995) and Kaemmer et al. (1997). Several DNA markers for bananas have been reported. These include restriction fragment length polymorphisms (RFLP) (Gawel and Jarret 1991; Jarret et al. 1992; Ge et al. 2005), variable number of tandem repeats (VNTR) polymorphisms (Kaemmer et al. 1992; Bhat et al. 1995), microsatellite DNA (Creste et al. 2003; Buhariwalla et al. 2005), random amplified polymorphic DNA (RAPD) (Onguso et al. 2004; Martin et al. 2006; Ray et al. 2006), amplified fragment length polymorphisms (AFLP) (Wong et al. 2001; Ude et al. 2002) and inter-simple sequence repeats (Ray et al. 2006). In general, hybridization-based techniques, e.g. RFLP, are straightforward but relatively time-consuming, especially when dealing with a large number of genotypes. In order to overcome this drawback, sequence-tagged microsatellite site (STMS) markers based on VNTR polymorphism of microsatellites, which are simple sequence repeats, have been developed. This technique is based on the polymerase chain reaction (PCR) and non-radioactive methods (Kaemmer et al. 1997). Using the mapping approach to compare the efficiency of different techniques, STMS markers have been shown to be more efficient than isozyme, RFLP and RAPD markers. Mansoor et al. (2005) reported that PCR-based methods can be used to detect banana bunchy top virus (BBTV) in banana, which will be useful for screening micropropagated banana plants for BBTV. On the other hand, a modified version of AFLP,

selective amplification of microsatellite polymorphic loci, has been adopted for fingerprinting somaclonal variants in banana (Giménez et al. 2005). Because of the diverse genetic background of the banana genome, it has been suggested that characterization of somaclonal variants may require more than one molecular marker (Asif and Othman 2005). Nevertheless, it is anticipated that these molecular markers may serve as important tools for analysis of genetic makeup, detection of desirable traits, somaclonal variants and germplasm preservation in breeding programs.

### 3 Identification of Banana Genes

The availability of genes responsible for useful traits is important for the elucidation of mechanisms that regulate plant growth and development, plant responses to biotic and abiotic stress at the molecular level, and for banana improvement using genetic engineering. During recent years, several attempts have been made to clone and to characterize genes from banana (Clendennen and May 1997; Medina-Suárez R et al. 1997; Drury et al. 1999; Gupta et al. 2006; Manrique-Trujillo et al. 2006). To isolate genes expressed in response to temperature stress, Santos and co-workers (2005) constructed two cDNA libraries from leaves of *M. accuminata* ssp. *burmannicoides* subject to low (5–25 °C) and high (25–45 °C) temperatures. Analysis of expressed sequence tags (ESTs) from the two libraries revealed that 10% of ESTs were commonly present in both tissues, while 42 and 48% were present specifically in tissue under low and high temperature stress, respectively (Santos et al. 2005). However, the identity of these genes and their functions are not known. In addition, several genes have been isolated from banana fruit and their identity has been determined putatively by sequence homology with published gene sequences deposited in the Genbank database. In this chapter, these genes are classified as ripening related, pathogenesis and stress related, and unknown function.

#### 3.1 Fruit Ripening

Bananas are climacteric fruits, which are characterized by low rates of respiration and ethylene production at the pre-climacteric stage during ripening, followed by a sudden upsurge at the climacteric and a sharp decline at the post-climacteric stage. This pattern of ethylene production during ripening has been attributed to autocatalytic production and inhibition (Yang and Hoffman 1984). Ripening is a complex process, which involves drastic changes in various biochemical events in the fruit. These include softening, loss of chlorophyll and increased yellowing of the peel, increased conversion of starch to sugars, and alteration of flavour and aroma. A large volume of evidence shows that ethylene plays a pivotal regulatory role in ripening (Lelièvre et al. 1997; Giovannoni 2001).

Bananas are highly perishable, with a short shelf life after harvest. Several common practices have been employed in attempts to prolong the shelf life of the fruit. These include refrigeration and controlled atmosphere storage, the use of ethylene absorbant, and surface coating and moist sawdust treatments (Abdullah et al. 1990), but the practices are either too costly or ineffective. It is important to understand the regulatory mechanism of ripening in order to manipulate and control the process. The current knowledge regarding the molecular biology of ripening has been derived primarily from work with tomato (Alexander and Grierson 2002). In recent years, major efforts have been devoted to elucidating the underlying mechanism of ripening at the molecular level (Giovannoni 2001). Genes associated with ripening have been isolated and characterized from several plant species, including peach (Trainotti et al. 2003), pear (Fonseca et al. 2004), melon (Hadfield et al. 2000), strawberry (Nam et al. 1999), citrus (Moriguchi et al. 1998), kiwifruit (Ledge and Gardner 1994) and tomato (Fei et al. 2004). In banana, several ripening-related genes have also been identified by differential expression of cDNA libraries in ripening fruits (Hill and ap Rees 1995; Clendennen and May 1997; Medina-Suárez et al. 1997; Drury et al. 1999; Manrique-Trujillo et al. 2007).

In our laboratory, we isolated 80 ESTs from a cDNA library constructed from the ripening fruit of *M. acuminata* cv. Williams (Liu 2000). Sequence analysis revealed a high homology of 30 ESTs with the known genes in the Genbank database. The distribution of these ESTs and their putative functions include four for  $\beta$ -1,3-glucanase, three each for pectate lyase and metallothionein (MT)-like protein (MLP) 2, and one each for UDPglucose pyrophosphorylase (UGPase), MLP 1, MLP 3, actin, isoflavone reductase, 1-aminocyclopropane-1-carboxylase (ACC) oxidase, O-methyltransferase, S-adenosylmethionine (SAM) synthetase, cytochrome P450 monooxygenase, 3-hydroxy-isobutryl-1-co-enzyme A hydrolase, malate synthase, glucosidase, vesicle transport protein, ferripyochelin-binding protein, ring-H<sub>2</sub>-binding protein, zinc finger protein, blue copper protein, putative cytidine 5'-triphosphate synthase, NAD-dependent isocitrate dehydrogenase and polyadenylate-binding protein 2 (Liu 2000). These enzymes/proteins are associated mainly with ethylene biosynthesis, carbohydrate metabolism, cell wall degradation, pathogenesis, senescence and stress. Some of these genes were further characterized and their roles were elucidated in banana by expression in fruit at different stages of ripening (see below).

### 3.1.1 Genes Associated with Ethylene Biosynthesis

Ethylene is a gaseous hormone that has been shown to play an important regulatory role in a wide range of plant physiological and developmental processes, including fruit ripening. The key enzymes involved in ethylene biosynthesis are SAM synthetase, ACC synthase and ACC oxidase. While SAM synthase catalyzes the conversion of methionine to SAM, the other two enzymes are re-

sponsible for the conversion of SAM to ACC and ACC to ethylene, respectively (Yang and Hoffman 1984).

Genes encoding ACC synthase and oxidase have been cloned in banana and a range of other plant species. Expression of these genes has also been characterized in banana fruit during ripening (López-Gómez et al. 1997; Liu et al. 1999; Pathak et al. 2003). Both enzymes are encoded by multigene families, and ACC synthase has been shown to be encoded by at least nine genes (Huang et al. 2006). In banana cv. Grand Naine, the level of ACC synthase transcripts was low or undetectable in flesh tissue of pre-climacteric fruit, but it surged transiently in climacteric fruit, followed by a rapid decline in post-climacteric fruit (Liu et al. 1999). This was similar to the pattern of endogenous ACC accumulation, ACC oxidase activity and ethylene production in fruit during ripening. However, the pattern of ACC oxidase transcript accumulation differed, as the transcript level increased markedly when fruit began to ripen and it remained high at post-climacteric stages. This differential pattern of accumulation between transcripts and enzyme activity was thought to be due partly to lower concentrations of cofactors such as ascorbate and iron (Liu et al. 1999). These findings are generally in line with the results of our study, in which both ACC synthase and oxidase genes were expressed temporally in peel and pulp of fruit of cv. Williams at different stages of ripening (Liu 2000). However, differences were also observed. We detected abundant ACC synthase transcripts and increased ACC accumulation in peel of climacteric and post-climacteric fruits. These fruits also showed high levels of the ACC oxidase activity, indicating that ACC oxidase did not play a major role in autocatalytic inhibition of ethylene during ripening.

### 3.1.2 *Genes Associated with Carbohydrate Metabolism*

Unripe banana fruit usually possesses large fractions of starch that constitute 20–25% of the pulp fresh weight. As ripening progresses, starch is rapidly converted to sugars, during which various genes are activated and their translation products are responsible for starch degradation and sugar synthesis. Although the physiological aspect of ripening in relation to carbohydrate metabolism is relatively well understood, knowledge regarding its molecular mechanism is virtually lacking.

To date, only a few ripening-related enzymes such as acid phosphatase (Turner and Plaxton 2001) and starch phosphorylase (Mota et al. 2002) and genes related to carbohydrate metabolism have been characterized in banana. One partial gene sequence for isoamylase-type starch-debranching enzyme (SDE) from banana fruit has been reported recently (Bierhals et al. 2004). SDE is involved in starch degradation by hydrolyzing  $\alpha$ -1,6-branches of amylopectin that, together with amylose, forms the starch. Interestingly, results showed little change in the levels of SDE transcript and protein during ripening. This has prompted the suggestion that pre-existing enzyme might be responsible

for starch hydrolysis. A gene for another starch-degrading enzyme  $\beta$ -amylase that catalyzes depolymerization of the  $\alpha$ -glucan chains has also been cloned (Nascimento et al. 2006). The levels of expression and enzyme activity of  $\beta$ -amylase in banana fruit increased with stage of ripening and, moreover, could be upregulated by exogenous application of ethylene. It was observed that the level of starch degradation was highly correlated with an increase in the  $\beta$ -amylase activity. Similar upregulation in transcript accumulation and enzyme activity during ripening has also been reported in  $\alpha$ -1,4-glucan-phosphorylase (Mainardi et al. 2006), an enzyme that is involved in both starch synthesis and degradation.

In sucrose synthesis, we have characterized a banana gene MWUGPA encoding UGPase (Pua et al. 2000), which is a two-way enzyme that catalyzes a reversible,  $Mg^{2+}$ -dependent reaction between glucose 1-phosphate and UDP-glucose. MWUGPA was shown to encode a single polypeptide of 467 amino acid residues. Southern analysis indicated the presence of more than one UGPase member in the banana genome. In RNA gel blot analysis, UGPase transcripts were detectable in both vegetative and reproductive organs, but those in vegetative organs were the least abundant. Furthermore, transcripts in pulp were considerably more than those in peel, although there was little change in transcript abundance in the respective fruit tissue during ripening, except in fruit at the climacteric stage. We also demonstrated that UGPase transcripts in fruit could be upregulated by exogenous application of ethylene, at 1 ppm, and sugars, especially sucrose and fructose (Pua et al. 2000). These results suggest the possible regulatory role of ethylene and sugars in UGPase expression in ripening fruit. Apart from UGPase, sucrose phosphate synthase (SPS) was also upregulated in ripening fruit (Nascimento et al. 1997). This enzyme catalyzes the conversion of UDP-glucose and fructose 6-phosphate to sucrose 6-phosphate, which can be further converted to sucrose by sucrose 6-phosphate phosphatase. Sucrose can also be synthesized from UDP-glucose and fructose catalyzed by sucrose synthase. It has been reported that over-expression of the SPS gene resulted in higher SPS and sucrose synthase activities and increased sucrose unloading in tomato fruit (Nguyen-Quoc et al. 1999).

### 3.1.3 Genes Associated with Cell Wall Proteins and Their Degradation

Expansins are the cellular proteins that are associated with cell wall loosening during fruit ripening. Wang et al. (2006) observed upregulated expression of expansin genes (*MaExp1* and *MaExp2*) in propylene pre-treated banana fruit, which was more tolerant to chilling injury during low temperature (7 °C) storage. It was suggested that chilling injury tolerance might be associated with increased ethylene production and expansin expression.

One common feature of ripening is fruit softening, which results from the increased activities of cell wall degrading enzymes (Brummell and Harpster



2001), including polygalacturonase (PG), pectin methylesterase and pectate lyase which are responsible for pectin depolymerization. In fruit pulp, the cell wall constitutes ~35% pectic polysaccharides. The role of these pectinases in fruit softening has been demonstrated in transgenic studies, where downregulation of PG (Smith et al. 1990), pectin methylesterase (Tieman et al. 1992) or pectate lyase (Jiménez-Bermúdez et al. 2002) in transgenic fruits resulted in a decrease in pectin depolymerization and cell wall solubilization, increase in solid substances and/or production of firmer fruits.

In banana, PG is the best characterized pectinase, which is encoded by a small gene family. Asif and Nath (2005) reported the isolation of four partial PG cDNAs from the ripe fruit pulp and these cDNAs were shown to express differentially; two were ripening related, while one was associated with senescence and the other expressed constitutively. Genes encoding pectate lyase have also been cloned and characterized in banana (Domínguez-Puigjaner et al. 1997; Pua et al. 2001; Marín-Rodríguez et al. 2003). We showed that there were two members (MWPL1 and MWPL2) of the pectate lyase gene in the banana genome. Expression of MWPLs differed both spatially and temporally during ripening. Whilst MWPL1 transcript was not detected in the vegetative and reproductive organs tested, MWPL2 was expressed predominantly in female flowers and ripening fruit peel and pulp (Pua et al. 2001). During ripening, both MWPL transcripts were not detected in pre-climacteric fruit, but they began to accumulate as ripening progressed and their levels remained high thereafter in overripe fruits, with a much greater MWPL2 than MWPL1 transcript. Expression of both members was also inducible by ethylene but dosage-dependent, with the highest expression in pre-climacteric fruit treated for 48 h with 50 ppm ethylene (Pua et al. 2000). In addition to ethylene, other growth regulators, such as abscisic acid, gibberellic acid and auxin, play a regulatory role in PG and pectate lyase in banana fruit (Lohani et al. 2004; Payasi et al. 2004), but the mechanism is not clear. In addition to PG and pectate lyases, ripening in banana has been associated with transcript accumulation of  $\beta$ -mannanase (Zhuang et al. 2006), which is a cell wall hydrolase responsible for the cleavage of mannan polymers.

### 3.1.4 Genes Associated With Fruit Flavour

During ripening, tissues develop a unique flavour that results, at least in part, from increased synthesis of organic acids (Grierson and Kader 1986). One banana gene cloned recently in our laboratory is malate synthase (MS) (Pua et al. 2003), which is a key enzyme responsible for the synthesis of malic acid. Both malic acid and citric acid are the predominant organic acids in banana fruit (John and Marchal 1995). MS is encoded by a small gene family in banana. The gene was shown to express specifically in fruit tissues, where abundant transcripts accumulated in post-climacteric fruits, especially in peel, but the level was not detected in pre-climacteric fruits (Pua et al. 2003). MS expression

was ethylene-inducible, suggesting that the climacteric rise of ethylene might be responsible for transcript accumulation in post-climacteric fruits.

The unique aroma and flavour of a fruit are characterized, at least in part, by the presence of specific volatile esters. A gene encoding alcohol acyltransferase (AAT) of 419 amino acid residues has been cloned recently from banana fruit (Beekwilder et al. 2004). AAT catalyzes the last step of ester formation by transacylation from an acyl-CoA to an alcohol. In banana, cinnamyl alcohol was the best substrate of AAT, but the role of AAT in fruit flavour is not clear. Results from a transgenic study showed that petunia overexpressing the AAT homologue from strawberry did not lead to altered volatile profile, but the tissues emitted the corresponding acetyl ester after the substrate isoamyl alcohol was added exogenously (Beekwilder et al. 2004). These findings suggest that the availability of alcohol substrate is important for genetic engineering of volatile ester formation in plants.

### 3.2 Pathogenesis and Stress-Related Genes

Several genes associated with plant defence and stress have been characterized in banana during recent years. These include  $\beta$ -1,3-glucanases (Liu 2000; Peumans et al. 2000), chitinases (Clendennen et al. 1998; Peumans et al. 2002), polyphenol oxidases (PPO) (Gooding et al. 2001) and MLPs (Liu et al. 2002). Both  $\beta$ -1,3-glucanase and chitinase have been considered as pathogenesis-related proteins, which have been shown to play a role in plant defence. In banana,  $\beta$ -1,3-glucanase is encoded by a multi-gene family and is expressed constitutively at a high level in fruit during ripening (Liu 2000). This may explain why several ESTs (five out of 80) isolated in our laboratory were  $\beta$ -1,3-glucanase. It was speculated that transcripts and the enzyme activity of  $\beta$ -1,3-glucanase present at high levels in banana fruit might not be associated with plant defence, but with fruit softening by degradation of unidentified cell-wall glucans (Peumans et al. 2000). However, the exact physiological role of this enzyme in ripening is not clear. Similarly, high levels of chitinase transcripts, with an unclear role, were also accumulated in banana fruit during ripening (Liu 2000). Peumans et al. (2002) observed an accumulation of class III chitinase protein during early fruit development, but the protein decreased progressively thereafter. This has prompted the suggestion that class III chitinase might serve as a supply of amino acids for the synthesis of ripening-related proteins.

Another banana gene implicated in plant defence is PPO, which is also associated with stress (Steffens et al. 1994; Thipyapong et al. 2004). PPO catalyzes an oxidative reaction, leading to the production of polyphenolic complexes that have been considered a major cause of brown discoloration in horticultural produce during handling, storage and processing. PPO in banana is encoded by a multi-gene family. The four PPO cDNAs isolated from the fruit expressed differentially in various organs, but the transcript level of all cD-



NAs was low in fruit tissues throughout the entire ripening period (Gooding et al. 2001). However, the physiological role of PPO in banana has yet to be elucidated.

MLPs are cysteine-rich polypeptides that are involved in metal detoxification and homeostasis. The five of 80 ESTs isolated in our laboratory were MLPs (Liu 2000), indicating that MLPs are abundant in banana fruit. We also characterized three members (MT2A, MT2B and MT3) of the MLP gene, whose expression varied spatially among the organs and temporally during fruit development and ripening (Liu et al. 2002). In fruit, MT2B was shown to express constitutively at all ripening stages, whereas MT2A expression decreased and MT3 increased with ripening. The response of the three MTs to ethylene and metals was also differential. While accumulation of MT2A transcripts was up-regulated by exogenous application of ethylene at 1 ppm but downregulated at 50 ppm, both MT2B and MT3 were not responsive to ethylene (Liu et al. 2002). However, MT2A and MT3 transcripts in fruit could be increased markedly by treatment of mature green fruit with metals such as  $\text{CdSO}_4$  (10–200  $\mu\text{M}$ ),  $\text{CuSO}_4$  (10–1,000  $\mu\text{M}$ ) and  $\text{ZnSO}_4$  (10–1,000  $\mu\text{M}$ ), although these had no effect on MT2B. MT regulation by metals has been well documented (Zhou and Goldsbrough 1994), but the regulatory role of ethylene in MT or MLP has not been reported previously. The results of our study, together with the isolation of MLPs from fruit tissues of banana (Clendennen and May 1997), apple (Reid and Ross 1997), kiwifruit (Ledger and Gardner 1994) and citrus (Moriguchi et al. 1998), clearly indicate that MLPs are involved in fruit development and ripening.

### 3.3 Genes of Unknown Function

A cDNA *MaExp1* encoding an expansin of 255 amino acids was isolated from ripening banana fruit (Trivedi and Nath 2004). As in the case of most banana genes described earlier, *MaExp1* expression was also inducible by exogenous ethylene and the transcript level in fruit increased with the progression of ripening. We also isolated and characterized a banana cDNA, *MAP450-1*, encoding a cytochrome P450 (Pua and Lee 2003). Cytochrome P450 is encoded by super-gene families. Judging from the sequence characteristics of *MAP450-1*, it was classified as CYP71N1, which is a member of the new CYP71 sub-family. *MAP450-1* was expressed specifically in fruit, where transcripts were detected only in ripe tissues. It was also shown that transcript accumulation in pre-climacteric fruit could be upregulated by exogenous ethylene, whereas expression in ripe fruit treated with exogenous sucrose, but not glucose, was downregulated (Pua and Lee 2003). These results clearly show that both genes are ripening related and ethylene inducible, but their physiological function in banana fruit is not clear.

## 4 Tissue Culture

The use of tissue culture techniques for vegetative propagation of bananas is a routine practice in many commercial nurseries. Table 1 summarizes the different pathways through which plants can be regenerated from shoot tip, callus, cell suspension and protoplast cultures via organogenesis or somatic embryogenesis.

### 4.1 Shoot Tip and Meristem Cultures

Shoot tips and meristems originated from suckers are the common explants used for banana micropropagation, which comprises various stages as described by Israel et al. (1995). Cultured shoots are usually induced to proliferate in MS-based medium (Murashige and Skoog 1962) with appropriate growth regulators, followed by induction of root formation from shoots and acclimatization of plants in soil. The rate of shoot proliferation is the most important factor of micropropagation. Madhulatha et al. (2004) reported that a liquid pulse treatment of shoot tip explants with cytokinins [ $50 \text{ mg l}^{-1}$  benzyladenine (BA) +  $50 \text{ mg l}^{-1}$  kinetin for 60 min] markedly increased the rate of shoot proliferation up to 14 shoots per explant. Similar treatment with auxins [ $100 \text{ mg l}^{-1}$   $\alpha$ -naphthaleneacetic acid (NAA) with  $100 \text{ mg l}^{-1}$  indole-3-butyric acid (IBA) for 60 min] also promoted root formation. It has been estimated that close to 1000 uniform plants can be produced from one single sucker within 10 months. Although the cost of setting up the tissue culture facilities is relatively expensive, the cost can be reduced by 90% using natural light and replacing some medium ingredients with low-cost alternatives, as demonstrated by Kodym and Zapata-Arias (2001).

Plants derived from shoot tip culture have been shown to perform equally well or even better than those from conventional vegetative propagation under field conditions (Drew and Smith 1990; Vuylsteke and Ortiz 1996). Although plants originated from shoot tip culture are generally more stable genetically compared to those generated from callus, cell suspensions and protoplasts, somaclonal variation has been observed (Vuylsteke et al. 1996), ranging from 0 to 70%, depending on the genotype (Smith 1988; Vuylsteke et al. 1991). To date, the exact causes of somaclonal variation are not clear. Several factors that may be associated with somaclonal variation in banana have been reviewed (Israel et al. 1995; Sahijram et al. 2003). Bairu et al. (2006) recently reported that somaclonal variation in Cavendish banana is associated with an increased number of subcultures and multiplication rate. It has been reported that somaclonal variants are generally inferior to the original clone from which they are derived (Vuylsteke et al. 1996). However, Giménez et al. (2001) reported the selection of a variant CIEN BTA-03 from Cavendish subgroup cv. Williams resistant to yellow Sigatoka disease, indicating the feasibility of selecting novel variants with agronomically important traits through somaclonal variation.

Table 1. Morphogenic responses and plant regeneration from cultured explants, cells and protoplasts of various banana genotypes

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of explant/ tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
cv. Williams (AAA)	Rhizome	Tissue sections derived from regions close to the apical meristem grown on SIM gave rise to more adventitious shoots, which were differentiated from epidermal cells. Regenerated shoots formed roots on MS with 30 g l <sup>-1</sup> sucrose, 6.5 g l <sup>-1</sup> agar, 1 µM NAA and 1 µM BA.	Li et al. (2006)
cv. Nanjanagud Rasabale (AAB)	Leaf sheathes from shoot culture	Explants cultured in the dark in the following sequential order promoted shoot bud formation: liquid M1 medium containing 22.4 µM BA and 0.198 µM IBA → M1 with 9.84 µM IBA (2 weeks) → M1 with 8.96 µM BA and 0.198 µM IBA (4 weeks). Explants grown on M1 with 0.83–20.7 µM picloram for 6–8 weeks gave rise to protocorm-like structure, which developed into shoot after being transferred to M1 basal medium.	Venkatachalam et al. (2006)
cv. Nendran (AAA)	Meristem tips	Optimal shoot proliferation was obtained from explants grown on MS medium containing a combination of sucrose and glucose (1:1) at the concentration of 30 g l <sup>-1</sup> . Root formation was most efficiently induced on the medium with 2 mg l <sup>-1</sup> auxin (IBA + NAA, 1:1).	Madhulatha et al. (2006)
cv. Mas (AA)	Immature male flower-derived embryogenic cell suspension	Cell aggregates differentiated and developed into mature embryos at high frequencies after being transferred to liquid half-strength MS basal medium with 30 g l <sup>-1</sup> sucrose and 5–7 mg l <sup>-1</sup> BA. Embryos developed into plants on full strength MS containing 7 mg l <sup>-1</sup> BA.	Wong et al. (2006)
cv. Grande Naine (AAA), var. SF 265 (AA)	Cell suspension- derived protoplasts	Protoplasts were isolated from embryogenic cell suspension maintained on Ma <sub>2</sub> medium. Ma <sub>2</sub> supplemented with 0.5 µM zeatin and 9.0 µM 2,4-D was best for the formation of microcalli and embryogenic cell aggregates from protoplasts. The number of microcalli increased seven-fold when a filter in conjugation with nurse cells was used.	Assani et al. (2006)

Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of explant/ tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
cv. Rajeli (AAB) Male flower primordia	Shoot tips from suckers	Shoot tips grown on MS medium with 8.9 $\mu\text{M}$ BA, 222.06 $\mu\text{M}$ AH and 3% sucrose were induced to form multiple shoots after 3–4 weeks. Individual shoots were rooted on MS medium with 5.37 $\mu\text{M}$ NAA and 1% sucrose. Explants were induced to form callus on MS medium with 18.1 $\mu\text{M}$ 2,4-D, 5.37 $\mu\text{M}$ NAA, 5.71 $\mu\text{M}$ IAA, 4.09 $\mu\text{M}$ biotin and 3% sucrose. Callus formed SEs after being transferred to medium with 4.52 $\mu\text{M}$ 2,4-D, 1 $\text{mg l}^{-1}$ biotin, 100 $\text{mg l}^{-1}$ malt extract and 4.5% sucrose. SE conversion was achieved on medium with 2.22 or 8.8 $\mu\text{M}$ BA and 3% sucrose, while hormone-free medium or medium with 5.37 $\mu\text{M}$ NAA was used for plant development.	Kulkarni et al. (2006)
cvs. Anamur 2, Dwarf Cavendish and Gazipasa 6	Shoot tips from suckers	Shoot proliferation was most efficient by culturing explants on MS medium containing 1–2.5 $\mu\text{M}$ TDZ and 1 $\mu\text{M}$ IAA. For rooting, hormone-free MS medium with 15 $\text{g l}^{-1}$ AC was recommended.	Gunbuk and Pekmezci (2006)
Plantain cv. CEMSA 3/4 (AAB)	Shoot tips from suckers	Shoot proliferation was optimal in MS medium containing 4.4 $\mu\text{M}$ meta-topolin using the temporary immersion system, in which 25–100 ml headspace per inoculum and 30 ml of medium per inoculum resulted in a multiplication rate of >13 in 28 days.	Roels et al. (2005)
	In vitro- grown shoots	Optimal rooting occurred in the modified MS medium containing 17.80–19.78 $\text{mmol l}^{-1}$ $\text{NO}_3\text{-N}$ as the sole N source supplemented with 30 $\text{g l}^{-1}$ sucrose, 0.5 $\text{mg l}^{-1}$ NAA and 0.5 $\text{mg l}^{-1}$ IBA.	Wu et al. (2005)
cv. Nendran (AAA)	Shoots from meristem tips	Pulse treatment of explants grown in MS basal medium with BA and K, each at 50 $\text{mg l}^{-1}$ , for 60 min resulted in optimal shoot proliferation. Treatment with NAA and IBA, each at 100 $\text{mg l}^{-1}$ , for 60 min was beneficial to root formation.	Madhulatha et al. (2004)

Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of explant/ tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
<i>M.b.</i> cv. Pisang Klutuk, Pisang Klutuk Wulung, Pisang Batu and Tani (BB)	Anthers	Explants grown for 6 months on medium containing MS salts, M vitamins (vit), 500 mg l <sup>-1</sup> CH, 73 mM sucrose, 4.4 µM BA, 2.3 µM NAA and 6 g l <sup>-1</sup> agarose formed androgenic callus. Callus gave rise to haploid plants after transfer to the same basal medium with 88 mM sucrose, 2.2 µM BA, 2.3 µM IAA and 7.5 g l <sup>-1</sup> agarose.	Assani et al. (2003)
<i>M.a.</i> cv. Mas (AA)	Immature male flowers	Explants formed embryogenic callus on M1 medium with 18 µM 2,4-D for 3 months, followed by 2–3 months on the same medium with 9 µM 2,4-D. On M4 medium with 0.8 µM BA, callus formed SEs, which developed into adventitious shoots in the dark. Shoots formed roots on hormone-free MS medium.	Jalil et al. (2003)
cv. Dwarf Brazilian (AAB)	Immature male flowers	PSE regenerated from explants grown on M1 medium for 2 months, followed by transfer to M1 with 200 mg l <sup>-1</sup> CH and 2 mg l <sup>-1</sup> proline. PSE gave rise to SSE after transfer to MS with 10% CW. SSE grown on MS with 5 mg l <sup>-1</sup> BA developed into mature embryos that further developed into plants after being transferred to hormone-free MS.	Khalil et al. (2002)
<i>M.a.</i> Cavendish (AAA) cv. Grande Naine and Gros Michel; plantain (AAB) cv. Currae Enano and Dominico; SF265, IRFA903 and Col49 (AA)	Cell suspension- derived protoplasts	Protoplasts cultured in medium (N6 salts + KM vit/organic acids/sugar alcohol + 117 mM sucrose + M vit + 0.4 M glucose + 0.5 mM MES 1.9 mM KH <sub>2</sub> PO <sub>4</sub> , 2.3 µM Z + 0.4 µM 2,4-D + 4.4 µM NAA) in the presence of feeder cells formed microcalli. Microcalli regenerated plants via somatic embryogenesis after transfer to medium with MS salts, M vit, 88 mM saccharose, 2.3 µM IAA, 2.2 µM BA and 7.5 g l <sup>-1</sup> Sea Plaque agarose.	Assani et al. (2002)
cv. FHIA-18 (AAAAB)	Immature male flower-derived	Cell suspension was initiated from embryogenic tissues and maintained in M2 medium. After transfer to M3 medium, cell suspensions	Kosky et al. (2002)

Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of explant/ tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
cv. Maçã (AAB) and cv. Lidi (AA)	embryogenic cell suspension	differentiated and developed into SEs. SEs developed into plants after being transferred to M4 medium.	Matsumoto et al. (2002)
	Protoplasts	Protoplasts isolated from embryogenic cell suspension of the cv. Maçã and non-embryogenic callus of the cv. Lidi were electrofused to form somatic hybrids. Fused protoplasts grown in D medium using nurse culture with a feeder layer formed SEs that developed into plantlets after transfer to semi-solid hormone-free MS medium, or medium with BA and NAA each at 2 µM	
cv. Rasthali (AAB)	In vitro- grown shoots	Shoot sections were cultured on MS with 9.05 µM 2,4-D, 1 µM Z and 1 mg l <sup>-1</sup> biotin for induction of embryogenic callus, which was used to initiate cell suspensions in M2 medium. Cell suspension gave rise to SEs after being transferred to half-strength medium with 10 µM Z. Sixty-six percent of SEs encapsulated in MS medium with 5% sodium alginate developed into plants.	Ganapathi et al. (2001b)
cv. Grande Naine (AAA)	Tissue culture- derived plants	Plants derived from embryogenic cell suspension and micropropagated shoots showed similar performance under field conditions.	Côte et al. (2000)
cv. Grande Naine (AAA)	Immature male flower-derived embryogenic cell suspension	Embryonic suspensions maintained in M2 medium developed into SEs after transfer to M3 medium. SEs developed into plants 3 months after transfer to M4 medium. The suspension exhibited five types of cell aggregate (types I–V), in which types II–IV belonged to the same development continuum of SE formation.	Georget et al. (2000)
False Horn plantain cv. Curraré and Curraré Enano (AAB)	Young female flowers	On M1 medium after 4–5 months, explants formed embryogenic tissue that was used to initiate cell suspensions in M2 medium. Cell suspensions developed into SEs after transfer to M3 medium. SEs grown on M4 medium developed into plants.	Grapin et al. (2000)

Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of ex- plant/tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
cv. Maçã (AAB)	Embryogenic callus-derived protoplasts	Protoplasts grown in D medium using nurse culture with a feeder layer formed SEs that developed into plants after transfer to semi-solid hormone-free MS medium, or medium with BA and NAA each at 2 µM.	Matsumoto and Oka (1998)
cv. Grand Naine (AAA)	Rhizome tissue	Explants formed embryogenic callus and/or SEs from adjacent cells of vascular tissues on (1) MS, (2) half-strength MS with 5 µM 2,4-D, 1 µM proline, 100 mg l <sup>-1</sup> CH, 40 mg l <sup>-1</sup> cysteine HCl, 10 µM ascorbate, 40 g l <sup>-1</sup> sucrose and 1.8 g l <sup>-1</sup> Gelrite, or (3) SH medium with 30 µM dicamba, B5 vit, 30 g l <sup>-1</sup> sucrose and 1.8 g l <sup>-1</sup> Gelrite.	Lee et al. (1997)
<i>M.a. ssp.</i> <i>malaccensis</i> (AA), cv. Grand Naine (AAA)	Immature zy- gotic embryos, male flower bud primordia	Five different media were used for callus induction and proliferation, SE maturation and germination, and plant growth for each <i>Musa</i> genotype.	Navarro et al. (1997)
False Horn plantain Agbaba (AAB)	Somaclonal variants tip culture from shoot	Three of four somaclonal variants performed poorly under field conditions compared to the original clone from which they were derived. These variants exhibited inflorescence degeneration, abnormal leaves and low yield.	Vuytsteke et al. (1996)
cv. Williams (AAA), plantain cv. Horn (AAB) and cv. Cachaco (ABB)	Micro-sections of shoot apical meristem	Shoot buds were regenerated from tissue sections grown on MS-mix (MS medium + v/aa mix) with 30 g l <sup>-1</sup> sucrose, 2.5 g l <sup>-1</sup> Phytigel, 1 µM NAA and 1 µM BA. Shoots were multiplied on MS-mix with 10 µM BA and 1 µM IAA. Explants grown on MS-mix with 10 µM dicamba and 1 µM IAA gave rise to embryogenic callus capable of forming plants.	Okole and Schulz (1996)
cv. Grand Naine (AAA)	Immature male flowers	Explants grown on M1 medium formed embryogenic callus after 5–6 months. Callus was transferred to M2 medium to initiate cell suspensions. The latter began to form SEs after transfer to M3 medium for 20–30 days. SEs were transferred to M4 and M5 medium for germination, where 3–20% of SEs developed leaves and roots.	Côte et al. (1996)

Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of ex- plant/tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
False Horn plantain Agbagba (AAB)	Shoot tip culture	Culture was initiated from lateral buds and grown on MS medium with 20 mg l <sup>-1</sup> ascorbate, 0.18 mg l <sup>-1</sup> IAA, 4.5 mg l <sup>-1</sup> BA and 2 g l <sup>-1</sup> Gellan Gum. Shoots were rooted in MS medium with 0.19 mg l <sup>-1</sup> NAA and 0.23 mg l <sup>-1</sup> BA. In vitro-grown plants grew more vigorously and taller than conventional sucker propagated plants, but there was no difference in fruit yield.	Vuylssteke and Ortiz (1996)
French Sombre plantain (AAB)	Young male flowers	Explants grown on M1 medium formed callus after 2–3 months. Friable callus was transferred to M2 medium to initiate cell suspensions. After the suspensions were transferred to M3 medium for 75 days, 40% of the suspensions developed SEs.	Grabin et al. (1996)
<i>M.a.</i> (AAA) cv. Grande Naine and Yangambi; plantain cv. French Plantain and Mysore (AAB) and Pelipita (ABB) cv. Bluggoe (AAB)	Immature male flowers       Cell suspensions	Explants grown on M1 medium gave rise to SEs after 3–5 months. SEs were germinated on M4 medium with reduced BA (0.22 µM) and developed in plants after transfer to M5 medium. The multiplication rate of SEs reached 40 with more than 6000 SEs after 6 months by subculturing in a temporary immersion system.  Protoplasts isolated from cell suspension were grown in half MS medium with 5 µM 2,4-D, 5% mannitol and 2 g l <sup>-1</sup> Gelrite with nurse culture (cell suspension). While 50% of protoplasts showed initial cell division, 20–40% formed microcalli. Microcalli were transferred to hormone-free medium, where they developed SEs and plants after 3 months.	Escalant et al. (1994)       Panis et al. (1993)
<i>M.a.</i> ssp. <i>malaccensis</i> and ssp. <i>burmannicoides</i>	Immature zygotec embryos	Explants formed callus on MM1 medium after 3 months. Callus was transferred to liquid MM1 medium with 126 mM sucrose to initiate cell suspensions, which gave rise to SEs after 8–12 weeks. SEs were also induced by culturing cell suspensions on semi-solid	Marroquin et al. (1993)



Table 1. (continued)

<i>Musa</i> species/ cultivar (group) <sup>a</sup>	Source of ex- plant/tissue/cell	Morphogenesis/growth/medium <sup>b</sup>	Reference
cv. Williams, Dwarf Cavendish and Grand Naine (AAA)	Tissue culture (TC)-derived plants	M1 medium. SEs were germinated on MM1 medium with 0.22 µM BA and 1.14 µM IAA.	Robinson et al. (1993)
<i>M.b.</i> and <i>M.a.</i> ssp. <i>microcarpa</i>	Immature zygotic embryos (45-day-old)	Under field conditions, TC plants were taller and thicker than conventional sucker-derived plants. The former also showed a higher annual yield of 13–19.4% compared to the latter over three crop cycles. Explants grown on modified M1 medium with 7.5 µM picloramformed callus after 3 months. Embryogenic cell suspensions were initiated from 5- to 17-week-old callus in liquid M1 medium. SEs were germinated on semi-solid M1 medium with 0.22 µM BA and 1.14 µM IAA, and developed into plants after transfer to hormone-free medium.	Marroquin et al. (1993)

<sup>a</sup> *M. a. Musa acuminata*; *M. b. Musa balbisiana*

<sup>b</sup> Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2ip, N<sup>6</sup>-(2-isopentenyl)adenine; AC, activated charcoal; AH, adenine hemisulphate; BA, benzyl adenine; CH, casein hydrolysate; CW, coconut water; dicamba, 3,6-dichloro-2-methoxybenzoic acid; D medium, half-strength MS macro-salts, MS micro-salts and vitamins, 10 mg l<sup>-1</sup> ascorbate, 5 µM 2,4-D, 20 g l<sup>-1</sup> sucrose and 0.55 mM-mannitol; glu, glutamine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; K, kinetin; KM, Kao and Michayluk 1975; M vitamins, Morel and Wetmore 1951; M1, MS salts and vitamins, 4.1 µM biotin, 5.7 µM IAA, 18.1 µM 2,4-D, 5.4 µM NAA, 90 mM sucrose and 7 g l<sup>-1</sup> agarose (Côte et al. 1996); M2, M1 salts and vitamins, 680 µM glu, 100 mg l<sup>-1</sup> malt extract, 4.5 µM 2,4-D and 130 mM sucrose; M3, SH salts, M1 vitamins, 680 µM glu, 2 mM proline, 100 mg l<sup>-1</sup> malt extract, 1.1 µM NAA, 0.23 µM Z, 0.46 µM K, 0.69 µM 2ip, 130 mM sucrose and 29.2 mM maltose; M4, MS salts, M vitamins, 2–3 µM BA, 1.1 µM IAA, 87 mM sucrose and 2 g l<sup>-1</sup> Gelrite; M5, same as M4 medium but without BA and NAA; Ma<sub>2</sub>, MS salts with 4.1 µM biotin, 4.5 µM 2,4-D, 680 µM glutamine, 100 mg l<sup>-1</sup> malt extract and 130 mM sucrose; MES, 2-(N-morpholino) ethanesulfonic acid; MM1, MS salts with half macro-elements, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, M vitamins, 7.5 µM picloram and 2 g l<sup>-1</sup> Gelrite; MS, Murashige and Skoog (1962) medium; N6, Chu et al. 1975; NAA, α-naph-thaleneacetic acid; picloram, 4-amino-3,5,6-trichloropicolinic acid; PSE, primary somatic embryos; SIM, shoot induction medium (MS salts, 40 g l<sup>-1</sup> sucrose, 6.5 g l<sup>-1</sup> agar, 10-µM BA, 50-µM K, 1-µM IAA and vit/amino acid mixture); SSE, secondary somatic embryos; SEs, somatic em-bryos; v/aa mix (mg l<sup>-1</sup>): thiamine HCl (0.4), pyridoxine-HCl (0.2), calcium pantothenate (0.03), nicotinic acid (0.2), folic acid (0.006), glycine (2), ascorbate (20), *p*-aminobenzoic acid (0.01), cysteine-HCl (10), riboflavin (0.004), myo-inositol (100), adenine sulphate (150) and L-tyrosine (100); SH, Schenk and Hildebrandt (1972) medium; TDZ, thidiazuron; Z, zeatin

In addition to micropropagation, cryopreservation of meristems has been used for germplasm preservation and the production of virus-free plants. In bananas, the cryopreservation protocol developed by Panis et al. (1996, 2000) may be useful for long-term storage of banana germplasm. Cryopreservation, which allows survival of only a small number of meristematic cells (Helliot et al. 2003), has also been shown to improve the efficiency in eliminating banana streak virus and cucumber mosaic virus from bananas (Helliot et al. 2002).

## 4.2 Somatic Embryogenesis and Androgenesis

Banana plants can also be regenerated from cultured cells via somatic embryogenesis (SE). This pathway is considered to be more efficient than that via organogenesis, as the former generally does not require the rooting step and plant production can be scaled up using bioreactors (Kosky et al. 2002). Somatic embryos can also be used for the production of synthetic seed (Ganapathi et al. 2001b), which facilitates handling, transportation and storage. The regeneration protocols via SE reported for various banana genotypes are quite similar (see Table 1). In general, explants are cultured on semi-solid MS-based medium supplemented with auxins to induce formation of embryogenic callus, which is used to initiate cell suspensions. Embryogenic cells are then induced to differentiate into embryos in an appropriate medium containing a combination of auxin and cytokinin. Embryos germinate and develop into plants after transfer to hormone-free semi-solid medium. Banana plants regenerated from cultured cells via SE have been shown to possess growth habits similar to those propagated from shoot tip culture and rhizomes under field conditions (Kosky et al. 2006).

The use of appropriate explants for the initiation of embryogenic callus is crucial for the success of plant regeneration via SE. Although embryogenic cells can be initiated from explants with no meristematic cells, such as rhizomes or basal leaf sheaths (Novak et al 1989; Lee et al. 1997; Venkatachalam et al. 2006), tissues with meristematic cells have been employed most commonly. These tissues include immature zygotic embryos (Escalant and Teissin 1989; Marroquin et al. 1993; Navarro et al. 1997), immature male flowers (Grapin et al. 1996; Khalil et al. 2002; Kosky et al. 2002; Jalil et al. 2003; Kulkarni et al. 2006) and female flowers (Grapin et al. 2000). However, the formation of embryogenic cells appears to be associated with explant age. In male flowers, the cluster positions between 3–4 and 11 have been shown to be most effective in embryogenic cell induction as compared to lower and higher flower clusters (Escalant et al. 1994; Jalil et al. 2003).

Plant regeneration from banana protoplasts via SE has also been reported (Panis et al. 1993; Matsumoto and Oka 1998; Assani et al. 2001). Protoplasts isolated from embryogenic calli or cell suspension can be induced to divide at high frequencies and to form embryogenic microcolonies. The use of high protoplast density, in conjunction with feeder cells, is crucial. It has been re-

ported that the mitotic activity of banana protoplasts can be induced by the presence of nurse cell culture (Assani et al. 2006). Plant regeneration is also affected by the type of materials used as a source of protoplasts. Assani et al. (2002) reported that protoplasts isolated from leaves and calli did not divide, but those from embryogenic cell suspension divided and differentiated into embryos. It was noted that the efficiency of somatic embryo formation varied with genotype. The most efficient genotype was the diploid SF265 (AA), which gave rise to most abundant somatic embryos, followed by the triploids Dominico (AAB) and Grande Naine (AAA). The development of efficient systems for plant regeneration from protoplasts is important for somatic hybrid production using protoplast fusion, which may offer an alternative approach to breeding to produce novel banana hybrids. The feasibility of producing somatic hybrids in banana has been demonstrated by Matsumoto et al. (2002), who reported five hybrids resulting from the electrofusion of protoplasts of Maçã (AAB) and Lidi (AA) bananas. These hybrids were verified by RAPD and cytometric ploidy analyses. Although cell fusion can be achieved by both electrofusion and polyethylene glycol treatment, the former has been shown to be more efficient than the latter, with respect to mitotic activities, SE and plant regeneration (Assani et al. 2005).

A recent study demonstrated the production of haploid plants in four banana genotypes (Pisang klutuk, Pisang batu, Pisang Klutuk wulung and Tani) using anther culture (Assani et al. 2003). Anthers with microspores at the uninucleate stage, cultured in the dark on MS-based medium containing casein hydrolysate and appropriate concentrations of growth regulators (4.4  $\mu\text{M}$  BA and 2.3  $\mu\text{M}$  indole-3-acetic acid), formed callus after 4 months and androgenic embryos after 6 months. Androgenic embryos developed into plants after transfer to regeneration medium. The ploidy of haploid plants was determined using flow cytometry. The ability to produce haploid plants will be important for banana breeding in the future, as haploidy offers many advantages, including the generation of homozygous lines, as reviewed by Hu and Guo (1999).

## 5 Genetic Transformation

Banana breeding has encountered some inherent problems associated with the crop, such as low fertility and genetic variability, as mentioned previously. These problems can be circumvented by using genetic engineering, through which genes of interest can be transferred routinely to bananas. Using the well-established tissue culture system, attempts have been made to transform bananas in different laboratories over the last 10 years and transgenic plants have been generated using both particle bombardment and *Agrobacterium*-mediated transformation.

## 5.1 Particle Bombardment

Sági et al. (1994) were the first to report the successful transfer of the reported gene *uidA* encoding  $\beta$ -glucuronidase using electroporation into protoplasts isolated from embryogenic cells of the cooking banana cv. Bluggoe. The transgene was shown to express transiently in transformed cells in 1.8% of the treated protoplasts. In the following year, the same research group reported stable transformation and the production of transgenic banana plants using particle bombardment of embryogenic cell suspensions (Sági et al. 1995). A similar approach was also used to produce transgenic Cavendish banana cv. Grand Naine (Becker et al. 2000). In general, particle bombardment is carried out using embryogenic cell suspensions. After bombardment, cells are cultured in appropriate selection medium for embryo induction, which usually takes several months. Both antibiotics and herbicide resistance have been used as selection markers, but hygromycin appears to be more effective than other markers (Sági et al. 1995). However, some antibiotics such as gentamicin and kanamycin may interfere with embryo germination (Becker et al. 2000). This can be circumvented by germination on selection-free medium, after which the selection pressure can be re-introduced at the rooting stage.

## 5.2 *Agrobacterium*-Mediated Transformation

In addition to particle bombardment, banana can be transformed using *Agrobacterium tumefaciens*, although banana is not the natural host of the bacterium. May et al. (1995) used the apical meristem dome and its underlying tissue wounded by bombardment with gold particles without coated DNA. After incubation on regeneration medium for 3 days, wounded tissues were inoculated for 30 min with the *A. tumefaciens* suspension containing 100  $\mu$ M acetosyringone. The tissues were co-cultivated with bacteria for 3 days on selection-free regeneration medium supplemented with acetosyringone, before transfer to selection medium containing kanamycin. Green regenerated shoots, that grew vigorously, were transferred to the selection rooting medium to induce root formation. These putative transformants were then verified by enzymatic assay for transgene activity and Southern analysis for the presence of transgenes. In this study, although stable transformants were obtained, chimerism was also observed (May et al. 1995). A similar transformation protocol has been used to produce transgenic bananas of the cv. Rasthali by co-cultivation of embryogenic cell suspensions with *A. tumefaciens* (Ganapathi et al. 2001a). A total of 200 putative transformants were selected based on herbicide resistance, 16 of which were confirmed to be transformed by the presence of  $\beta$ -glucuronidase (GUS) activity in fruit and other tissues and Southern analysis. Recently, Tripathi and co-workers (2005) reported the production of transgenic plantain cv. Agbagba by co-cultivation of apical shoot tips with *A. tumefaciens*.

The transformation frequency can be improved by the use of centrifugation or vacuum infiltration during inoculation and co-cultivation of explants with *Agrobacterium*. Khanna et al. (2004) reported a three- to four-fold increase in transient gene expression and stable transformation of banana by centrifugation of embryogenic cell suspensions co-cultivated with *A. tumefaciens* at 1,000 rpm for 5 min at room temperature. The increase may be associated with increased bacterial colonization on the surface of suspension cells, but the exact reason is not clear. The transformation frequency was not affected by heat shock treatment that, however, doubled the viability of suspension cells after inoculation (Khanna et al. 2004). Apart from centrifugation, vacuum infiltration is also beneficial for banana transformation. This was demonstrated by Acereto-Escoffié et al. (2005), who reported increased transformation by vacuum-infiltration for 4 min at 400 mm Hg of banana meristematic tissues with *Agrobacterium* suspension. Pei et al. (2005) also reported a high frequency of banana transformation by a combination of particle bombardment and *A. tumefaciens*-mediated transformation. A transformation frequency of 0.95% was achieved by first bombarding the banana tissues of cultured shoots with naked gold particles, incubated on regeneration medium for 3 days, followed by co-cultivated with *A. tumefaciens* in conjunction with vacuum-infiltration.

Transgenic plants are usually verified using molecular techniques such as DNA and/or RNA gel blot analysis. However, these methods are relatively time-consuming. PCR offers a fast approach for verification of transgenic plants, but it may not be specific, especially when dealing with putative primary transformants. This is because the primary transformants may contain *Agrobacterium* which carries the transgenes. Pérez-Hernández et al. (2006) recently reported an improved anchored PCR technique that amplified specific T-DNA border-containing genomic sequences in transgenic banana. The amplified band pattern generated from unique enzyme and primer combinations for each individual transgenic plant can serve as a fingerprint that allows for the identification of a particular transgenic plant or transformation event.

## 6 Factors Affecting Transgene Expression

Transgene expression in banana can be affected by the promoter. A comparative study on transient expression of the chimeric GUS gene driven by different promoters in banana showed that the maize ubiquitin promoter was more effective than the cauliflower mosaic virus (CaMV) 35S promoter, with or without the alfalfa mosaic virus untranslated region (UTR), and the Emu recombinant promoter (Sági et al. 1995). Schenk et al. (1999) reported a 1369-bp promoter from sugarcane bacilliform badnavirus (ScBV) that could direct near constitutive transgene expression in both banana and tobacco plants. Expression in banana was comparable to that conferred by the maize ubiquitin promoter. However, a 2105-bp promoter from Australian banana streak bad-

navirus (BSV) was found to confer stronger gene expression in banana than the ubiquitin promoter (Schenk et al. 2001). The activity of the former was seven-fold greater in leaf tissues and up to four-fold more in root and shoot tissues than the latter. Furthermore, the BSV promoter also conferred high transgene expression in other monocotyledons and dicotyledons. These results indicate that the ScBV and BSV promoters are efficient in directing gene expression in both monocots and dicots. The use of taro bacilliform badnavirus (TaBV) promoter also conferred near constitutive expression in banana, where expression was highest in the vascular tissue (Yang et al. 2003). In addition to ScBV and TaBV, the promoter activity of BBTv, which infects members of the genus *Musa*, has been evaluated. BBTv comprises at least six single-stranded DNA components, DNA-1 to -6, each of which are at least 1 kb in size (Burns et al. 1995). Low levels of expression of the green fluorescent protein (GFP) reporter gene, but not GUS, driven by different BBTv promoters derived from DNA-1 to -6, have been detected transiently in transformed banana cells, with DNA-4 and -5 giving the highest expression (Dugdale et al. 1998, 2000). Expression was localized mainly in the phloem and vascular-associated cells, indicating the possible role of these genes in BBTv transmission, which was shown to be phloem-limited (Magee 1939).

In the transformation of monocotyledons, the promoting effect of the 5' UTR, especially introns, in genes has been well documented (Vasil et al. 1989; Rethmeier et al. 1997), although its molecular mechanism is not clear. In banana cells, a marked increase in the promoter activity of BBTv DNA-6 was observed when an intron was inserted between the reporter gene and promoter (Dugdale et al. 2001). However, the magnitude varied considerably with the promoter. The use of rice actin and maize ubiquitin introns resulted in increased transgene activity by 300- and 100-fold, respectively, while the intron from the sugarcane small subunit of Rubisco increased expression by 10-fold. The maize alcohol dehydrogenase intron had no effect. Similar intron-mediated enhancement of promoter activity was observed in transgenic banana plants with the ubiquitin intron. The presence of the ubiquitin intron also enhanced the promoter activity of S1 and S2 of Taiwanese BBTv isolates, which conferred organ-specific expression in root meristems and trichomes (Hermann et al. 2001).

The development of a binary bacterial artificial chromosome (BIBAC) library is an important technique for introducing large fragments of DNA (up to 120 Kp) into the plant genome via *A. tumefaciens*-mediated transformation (Hamilton et al. 1996, 1999). This technology can not only transfer gene clusters, but also be used for gene identification. Recently, a BIBAC genomic library has also been constructed from the black Sigatoka-resistant banana cv. Tuu Gia (AA) (Ortiz-Vázquez et al. 2005). The library has been estimated to represent five times its haploid genome and each library clone carries the DNA insert of around 100 kb. The authors speculated that the BIBAC library clones could be directly transferred to a Sigatoka-susceptible cv. using *A. tumefaciens*, thus enabling identification of genes responsible for disease resistance.



## 7 Applications of Transgenic Plants

### 7.1 Disease and Pest Resistance

Banana cultivation has been threatened by a number of diseases and pests. The most important diseases are Panama wilt and Sigatoka disease caused by *Fusarium oxysporum* f. sp. *cubense* and *Mycosphaerella musicola*, respectively. The approach for controlling the diseases is to introduce resistant cultivars produced by conventional plant breeding methods, but the latter have several constraints, as mentioned previously (see Sect. 2). Similarly, nematodes are important pests of banana that can cause considerable losses. Control is usually achieved by periodic sprayings of nematicides, such as organophosphates and carbamates (Whitehead 1998), but the practice can result in environmental pollution and is hazardous to workers. In recent years, attempts have been made to control nematode diseases using genetic engineering. It has been reported that transgenic plants that expressed the rice gene encoding cystatin, which is a cysteine proteinase inhibitor, under the control of maize ubiquitin promoter, are more resistant to burrowing nematode (*Radopholus similis*) compared to non-transformed control (Atkinson et al. 2004).

A similar transgenic approach has been employed to control Panama wilt and Sigatoka disease. Chakrabarti and co-workers (2003) reported that transgenic banana expressing a synthetic substitution analogue of magainin, a protein from skin secretions of *Xenopus laevis*, was more resistant to both *F. oxysporum* f. sp. *cubense* and *M. musicola*. Similar resistance to the fungal pathogen has been reported in transgenic banana expressing chitinase and  $\beta$ -1,3-glucanase (Sreeramanan et al. 2006). Results of a recent study showed that banana plants that expressed a human lysozyme gene under the control of 35S promoter were also resistant to Panama wilt disease under field conditions (Pei et al. 2005). The degree of resistance correlated positively with transgene expression, with the two most resistant transgenic lines, H-67 and H-144, showing the highest gene expression.

### 7.2 Potential Applications

As in other fruit crops, it should be possible to improve banana by genetic engineering. Bananas have a relatively short shelf life, owing to their rapid ripening triggered by ethylene, which is a major cause of post-harvest losses. This problem may be alleviated by inhibition of ethylene production through downregulation of genes encoding ACC synthase and ACC oxidase, thereby prolonging the shelf life of banana, as demonstrated in tomato (Tucker et al. 2006) and melon (Chapter 2). In addition, carbohydrate metabolism in banana can be modulated by metabolic engineering, as in potato (Börnke et al. 2006).

In recent years, plants have been considered as an attractive system for the production of vaccines, antibodies and other therapeutic proteins, as their use

reduces health risks from pathogen contamination and product synthesis is more cost-effective compared to the conventional production system using fermentation and mammalian cells (Giddings et al. 2000; Chargelegue et al. 2001; Stoger et al. 2002). It has been shown that therapeutic proteins can be targeted and faithfully expressed in plants, although several limitations remain to be overcome (Kirk et al. 2005). Producing therapeutic proteins in banana has several advantages because it eliminates the need for costly and time-consuming processing (e.g. extraction and purification), syringes and needles, and sterile conditions. Furthermore, it allows direct administration without cooking, through which heat labile proteins may be denatured and destroyed. Sunil Kumar et al. (2005) recently reported the expression of hepatitis B surface antigen (HBsAg) in transgenic banana up to 38 ng per gram leaf fresh weight. Although the expression level remains low, this study has demonstrated the feasibility of expressing HBsAg, and possibly other novel therapeutic proteins and vaccines, in banana.

## 8 Conclusions

In the last 10 years, significant progress has been made in banana biotechnology. This includes the development of molecular markers for breeding and methodology for transgenic plant production, cloning and characterization of genes associated with fruit development and ripening. Furthermore, transgenic technology has also been used to produce novel bananas resistant to diseases and pests. Although reports on transgenic bananas with improved agronomic traits have been limited, the results of these studies indicate a considerable potential in using transgenic technology for the genetic improvement of banana. In order to achieve this, more genes responsible for various agronomically important traits have yet to be cloned and characterized.

As the size of the banana genome of 500–600 Mbp is relatively small, a concerted effort has been undertaken since 2001 by an international Global *Musa* Genomics Consortium to sequence the genome (see <http://www.musagenomics.org/index.php?page=beyond>). It is anticipated that this sequencing will be completed within 5 years. This sequencing project will facilitate the identification of genes associated with agronomic traits, such as disease and pest resistance, environmental stress and fruit yield and quality, which will be useful in future endeavours relating to the improvement of bananas.

## References

- Abdullah H, Lizada MCC, Tan SC, Pantastico ErB, Tongdee SC (1990) Storage of banana. In: Hassan A, Pantastico EB (eds) Fruit development, postharvest physiology, handling and marketing in ASEAN. ASEAN Food Handling Bureau, Kuala Lumpur, pp 44–64



- Acereto-Escoffié POM, Chi-Manzanero BH, Echeverría S, Grijalva R, Kay AJ, González-Estrada T, Castaño E, Rodríguez-Zapata LC (2005) *Agrobacterium*-mediated transformation of *Musa acuminata* cv. "Grand Nain" scalps by vacuum infiltration. *Sci Hort* 105:359–371
- Aert R, Sági L, Volckaert G (2004) Gene content and density in banana (*Musa acuminata*) as revealed by genomic sequencing of BAC clones. *Theor Appl Genet* 109:129–139
- Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *J Exp Bot* 53:2039–2055
- Asif MH, Nath P (2005) Expression of multiple forms of polygalacturonase gene during ripening in banana fruit. *Plant Physiol Biochem* 43:177–184
- Asif MJ, Othman RY (2005) Characterization of *Fusarium* wilt-resistant and *Fusarium* wilt-susceptible somaclones of banana cultivar Rastali (*Musa* AAB) by random amplified polymorphic DNA and retrotransposon markers. *Plant Mol Biol Rep* 23:341–349
- Assani A, Haïcour R, Wenzel G, Cote F, Bakry F, Foroughi-Wehr B, Ducreux G, Aguillar ME, Grapin A (2001) Plant regeneration from protoplasts of desert banana cv. Grande Naine (*Musa* spp., Cavendish sub-group AAA). *Plant Cell Rep* 20:482–488
- Assani A, Haïcour R, Wenzel G, Foroughi-Wehr B, Bakry F, Côte FX, Ducreux G, Ambroise A, Grapin A (2002) Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (*Musa* spp.). *Plant Sci* 162:355–362
- Assani A, Bakry F, Kerbellec F, Haïcour R, Wenzel G, Foroughi-Wehr B (2003) Production of haploids from anther culture of banana [*Musa balbisiana* (BB)]. *Plant Cell Rep* 21:511–516
- Assani A, Chabane D, Haïcour R, Bakry F, Wenzyl G, Foroughi-Wehr B (2005) Protoplast fusion in banana (*Musa* spp.): comparison of chemical (PEG: polyethylene glycol) and electrical procedure. *Plant Cell Tissue Organ Cult* 83:145–151
- Assani A, Chabane D, Foroughi-Wehr B, Wenzyl G (2006) An improved protocol for microcallus production and whole plant regeneration from recalcitrant banana protoplasts (*Musa* spp.). *Plant Cell Tissue Organ Cult* 85:257–264
- Atkinson HJ, Grimwood S, Johnston K, Green J (2004) Prototype demonstration of transgenic resistance to the nematode *Rodopholus similes* conferred on banana by a cystatin. *Transgenic Res* 13:135–142
- Bairu MW, Fennell CW, van Staden J (2006) The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa* AAA cv. 'Zelig'). *Sci Hort* 108:347–351
- Becker DK, Dugdale B, Smith MK, Harding RMJ, Dale JL (2000) Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv. 'Grand Nain' via microprojectile bombardment. *Plant Cell Rep* 19:229–234
- Beekwilder J, Alvarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester HJ, Aharoni A (2004) Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiol* 135:1865–1878
- Bhat KV, Bhat SR, Chandel KPS, Lakhnampaul S, Ali S (1995) DNA fingerprinting of *Musa* cultivars with oligodeoxyribonucleotide probes specific for simple repeat motifs. *Genet Anal* 12:45–51
- Bierhals JD, Lajolo FM, Cordenunsi BR, Nascimento JRO (2004) Activity, cloning and expression of an isoamylase-type starch-debranching enzyme from banana fruit. *J Agric Food Chem* 52:7412–7418
- Börnke F, Sonnewald U, Biemelt S (2006) Potato. In: Pua EC, MR Davey (eds) *Biotechnology in agriculture and forestry*, vol 59. *Transgenic crops IV*. Springer, Berlin Heidelberg New York
- Brummell DA, Harpster MH (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol Biol* 77:311–340
- Buhariwalla HK, Jarret RL, Jayashree B, Crouch JH, Ortiz R (2005) Isolation and characterization of microsatellite markers from *Musa balbisiana*. *Mol Ecol Notes* 5:327–330
- Burns TM, Harding RM, Gale JL (1995) The genome organization of banana bunchy top virus: analysis of six ssDNA components. *J Gen Virol* 76:1471–1482
- Chakrabarti A, Ganapathi TR, Mukherjee PK, Bapat VA (2003) MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. *Planta* 216:587–596
- Chargelegue D, Obregon P, Drake PMW (2001) Transgenic plants for vaccine production: expectations and limitations. *Trends Plant Sci* 6:495–496

- Chu CC, Wang CC, Sun CS (1975) Establishment of efficient medium for anther culture of rice through comparative experiments on the nitrogen source. *Sci Sin* 17:659–668
- Clendennen SK, May GD (1997) Differential gene expression in ripening banana fruit. *Plant Physiol* 115:463–469
- Clendennen SK, Lopez-Gomez R, Gomez-Lim M, Arntzen CJ, May GD (1998) The abundant 31-kilodalton banana pulp protein is homologous to class-III acidic chitinases. *Phytochemistry* 47:613–619
- Côte FX, Domergue R, Monmarson S, Schwendiman J, Teisson C, Escalant JV (1996) Embryogenic cell suspension from the male flower of *Musa* AAA cv. Grand Nain. *Physiol Plant* 97:285–290
- Côte FX, Folliot M, Domergue R, Dubois C (2000) Field performance of embryogenic cell suspension-derived banana plants (*Musa* AAA, cv. Grande Naine). *Euphytica* 112:245–251
- Creste S, Tulmann Neto A, Silva SO, Figueira A (2003) Genetic characterization of banana cultivars (*Musa* spp.) from Brazil using microsatellite markers. *Euphytica* 132:259–268
- D'Hont A (2005) Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. *Cytogenet Genome Res* 109:27–33
- D'Hont A, Paget-Goy A, Escoute J, Carreel F (2000) The interspecific genome structure of cultivated banana, *Musa* spp. revealed by genomic DNA in situ hybridization. *Theor Appl Genet* 100:177–183
- Domínguez-Puigjaner E, Llop I, Vendrell M, Prat S (1997) A cDNA clone highly expressed in ripe banana fruit shows homology to pectate lyase. *Plant Physiol* 114:1071–1076
- Drew RA, Smith MK (1990) Field evaluation of tissue-cultured bananas in south-eastern Queensland. *Aust J Exp Agric* 30:569–574
- Drury R, Hörtensteiner S, Donnison I, Bird CR, Seymour GB (1999) Chlorophyll catabolism and gene expression in the peel of ripening banana fruits. *Physiol Plant* 107:32–38
- Dugdale B, Beetham PR, Becker DK, Harding RM, Dale JL (1998) Promoter activity associated with the intergenic regions of banana bunchy top virus DNA-1 to -6 in transgenic tobacco and banana cells. *J Gen Virol* 79:2301–2311
- Dugdale B, Becker DK, Beetham PR, Harding RM, Dale JL (2000) Promoters derived from banana bunchy top virus DNA-1 to -5 direct vascular-associated expression in transgenic banana (*Musa* spp.). *Plant Cell Rep* 19:810–814
- Dugdale B, Becker DK, Harding RM, Dale JL (2001) Intron-mediated enhancement of the banana bunchy top virus DNA-6 promoter in banana (*Musa* spp.) embryogenic cells and plants. *Plant Cell Rep* 20:220–226
- Escalant JV, Teissin C (1989) Somatic embryogenesis and plants from immature zygotic embryos of the species *Musa acuminata* and *Musa balbisiana*. *Plant Cell Rep* 7:665–668
- Escalant JV, Teissin C, Côte FX (1994) Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (*Musa* spp.). *In Vitro Cell Dev Biol* 30P:181–186
- Fei Z, Tang X, Alba RM, White JA, Ronning CM, Martin GB, Tanksley SD, Giovannoni JJ (2004) Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. *Plant J* 40:47–59
- Fonseca S, Hackler L, Zvara A, Ferreira S, Baldé A, Dudits D, Pais MS, Puskás LG (2004) Monitoring gene expression along pear fruit development, ripening and senescence using cDNA microarrays. *Plant Sci* 167:457–469
- Ganapathi TR, Higgs NS, Balint-Kurti PJ, Arntzen CJ, May GD, Van Eck JM (2001a) *Agrobacterium*-mediated transformation of embryogenic cell suspensions of the banana cultivar Rasthali (AAB). *Plant Cell Rep* 20:157–162
- Ganapathi TR, Srinivas L, Suprasanna P, Bapat VA (2001b) Regeneration of plants from alginate-encapsulated somatic embryos of banana cv. Rasthali (*Musa* spp. AAB group). *In Vitro Cell Dev Biol-Plant* 37:171–181
- Gawel NJ, Jarret RL (1991) Chloroplast DNA restriction fragment length polymorphism (RFLPs) in *Musa* spp. *Theor Appl Genet* 81:783–786
- Ge XJ, Liu MH, Wang WK, Schaal BA, Chiang TY (2005) Population structure of wild bananas, *Musa balbisiana*, in China determined by SSR fingerprinting and cpDNA PCR-RFLP. *Mol Ecol* 14:933–944

- Georget F, Domergue R, Ferrière N, Côte FX (2000) Morphological study of the different constituents of a banana (*Musa* AAA, cv. Grande Naine) embryogenic cell suspension. *Plant Cell Rep* 19:748–754
- Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol* 18:1151–1155
- Giménez C, De García E, De Enrech NX, Blanca I (2001) Somaclonal variation in banana: cytogenetic and molecular characterization of the somaclonal variant CIEN BTA-03. *In Vitro Cell Dev Biol-Plant* 37:217–222
- Giménez C, Palacios G, Colmenares M, Kahl G (2005) SAMPL: a technique for somaclonal variation fingerprinting in *Musa*. *Plant Mol Biol Rep* 23:263–269
- Giovannoni J (2001) Molecular biology of fruit maturation and ripening. *Annu Rev Plant Physiol Plant Mol Biol* 52:725–749
- Gooding PS, Bird C, Robinson SP (2001) Molecular cloning and characterization of banana fruit polyphenol oxidase. *Planta* 213:748–757
- Grabin A, Schwendiman J, Teisson C (1996) Somatic embryogenesis in plantain banana. *In Vitro Cell Dev Biol* 32:66–71
- Grabin A, Ortiz JL, Lescot T, Côte FX (2000) Recovery and regeneration of embryogenic cultures from female flowers of False Horn Plantain. *Plant Cell Tissue Organ Cult* 61:237–244
- Grierson D, Kader AA (1986) Fruit ripening and quality. In: Atherton JG, Rudich J (eds) *The tomato crops*. Chapman Hall, London, pp 241–280
- Gunbuk H, Pekmezci M (2006) In vitro propagation of banana (*Musa* spp.) using thidiazuron and activated charcoal. *Acta Agric Scand Sec B* 56:65–69
- Gupta SM, Srivastava S, Sane AP, Nath P (2006) Differential expression of genes during banana fruit development, ripening and 1-MCP treatment: presence of distinct fruit specific, ethylene induced and ethylene repressed expression. *Postharvest Biol Technol* (in press)
- Hadfield KA, Dang T, Guis M, Pech JC, Bouzayen M, Bennett AB (2000) Characterization of ripening-regulated cDNAs and their expression in ethylene-suppressed Charentais melon fruit. *Plant Physiol* 122:977–983
- Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proc Natl Acad Sci USA* 93:9975–9979
- Hamilton CM, Frary A, Xu Y, Tanksley SD, Zhang HB (1999) Construction of tomato genomic DNA libraries in a binary-BAC (BIBAC) vector. *Plant J* 18:223–229
- Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Erison E (2002) Cryopreservation for elimination of cucumber mosaic and banana streak viruses from banana (*Musa* spp.). *Plant Cell Rep* 20:1117–1122
- Helliot B, Swennen R, Poumay Y, Erison E, Lepoivre P, Panis B (2003) Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. *Plant Cell Rep* 21:690–698
- Hermann SR, Becker DK, Harding RM, Dale JL (2001) Promoters derived from banana bunchy top virus-associated components S1 and S2 drive transgene expression in both tobacco and banana. *Plant Cell Rep* 20:642–646
- Hill SA, ap Rees T (1995) The effect of glucose on the control of carbohydrate metabolism in ripening bananas. *Planta* 197:313–323
- Hu H, Guo X (1999) In vitro induced haploids in plant genetics and breeding. In: Soh WY, Bhowjani SS (eds) *Morphogenesis in plant tissue cultures*. Kluwer, Dordrecht, pp 329–361
- Huang FC, Do YY, Huang PL (2006) Genome organization of a diverse ACC synthase gene family in banana and expression characteristics of the gene member involved in ripening of banana fruits. *J Agric Food Chem* 54:3859–3868
- Israel Y, Lahav E, Reuveni O (1995) In vitro culture of banana. In: Gowen S (ed) *Bananas and plantains*. Chapman & Hall, London, pp 147–178
- Jain SM (2005) Major mutation-assisted plant breeding programs supported by FAO/IAEA. *Plant Cell Tissue Organ Cult* 82:113–123
- Jalil M, Khalid N, Othman RY (2003) Plant regeneration from embryogenic suspension cultures of *Musa acuminata* cv. Mas (AA). *Plant Cell Tissue Organ Cult* 75:209–214

- Jarret RL, Gawel NJ (1995) Molecular markers, genetic diversity and systematics in *Musa*. In: Gowen S (ed) Bananas and plantains. Chapman & Hall, London, pp 66–83
- Jarret RL, Gawel NJ, Whittmore A, Sharrock S (1992) RFLP-based phylogeny of *Musa* species in Papua New Guinea. *Theor Appl Genet* 84:579–584
- Jiménez-Bermúdez S, Redondo-Nevaldo J, Muñoz-Blanco J, Caballero JL, López-Aranda JM, Valpuesta V, Pliego-Alfaro F, Quesada MA, Mercado JA (2002) Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiol* 128:751–759
- John P, Marchal J (1995) Ripening and biochemistry of the fruit. In: Gowen S (ed) Bananas and plantains. Chapman & Hall, London, pp 432–467
- Kaemmer D, Afza R, Weising K, Kahl G, Novak FJ (1992) Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp.). *Bio/Technology* 10:1030–1035
- Kaemmer D, Fischer D, Jarret RL, Baurens F-C, Grapin A, Dambier D, Noyer J-L, Lanaud C, Kahl G, Lagoda PJL (1997) Molecular breeding in the genus *Musa*: a strong case for STMS marker technology. *Euphytica* 96:49–63
- Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta* 126:105–110
- Khalil SM, Cheah KT, Perez EA, Gaskill DA, Hu JS (2002) Regeneration of banana (*Musa* spp. AAB cv. Dwarf Brazilian) via secondary embryogenesis. *Plant Cell Rep* 20:1128–1134
- Khanna H, Becker D, Kleidon J, Dale J (2004) Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). *Mol Breed* 14:239–252
- Kirk DD, McIntosh K, Walmsley AM, Peterson RKD (2005) Risk analysis for plant-made vaccines. *Transgenic Res* 14:449–462
- Kodym A, Zapata-Arias FJ (2001) Low-cost alternatives for micropropagation of banana. *Plant Cell Tissue Organ Cult* 66:67–71
- Kosky RG, de Faria Silva M, Pérez LP, Gilliard T, Martínez B, Vega MR, Milian MC, Mendoza EQ (2002) Somatic embryogenesis of the banana hybrid cultivar FHIA-18 (AAAB) in liquid medium and scaled-up in a bioreactor. *Plant Cell Tissue Organ Cult* 68:21–26
- Kosky RG, Barranco LA, Pérez LP, Daniels D, Vega MR, de Faria Silva M (2006) Trueness-to-type and yield components of the banana hybrid cultivar FHIA-18 plants regenerated via somatic embryogenesis in bioreactor. *Euphytica* 150:63–68
- Kulkarni VM, Suprasanna P, Bapat VA (2006) Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli. *Curr Sci* 90:842–846
- Ledger SE, Gardner RC (1994) Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var. *deliciosa*). *Plant Mol Biol* 25:877–886
- Lee KS, Zapata-Arias FJ, Brunner H, Afza R (1997) Histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa* spp. *Plant Cell Tissue Organ Cult* 51:1–8
- Lelièvre JM, Latché A, Jones B, Bouzayen M, Pech JC (1997) Ethylene and fruit ripening. *Physiol Plant* 101:727–739
- Li J, Huang XL, Wei YR, Huang X, Li Z, Li XJ (2006) Histological analysis of direct organogenesis from micro-cross-sections of cultures of the banana. *Aust J Bot* 54:595–599
- Liu P (2000) Molecular and physiological study on fruit ripening in banana (*Musa acuminata*). PhD thesis, National University of Singapore, Singapore
- Liu P, Goh CJ, Loh CS, Pua EC (2002) Differential expression and characterization of three metallothionein-like protein genes during fruit ripening of banana (*Musa acuminata*). *Physiol Plant* 114:241–250
- Liu X, Shiomi S, Nakatsuka A, Kubo Y, Nakamura R, Inaba A (1999) Characterization of ethylene biosynthesis associated with ripening in banana fruit. *Plant Physiol* 121:1257–1265
- Lohani S, Trivedi PK, Nath P (2004) Changes in activities of cell wall hydrolases during ethylene-induced ripening in banana: effect of 1-MCP, ABA and IAA. *Postharvest Biol Technol* 31:119–126

- López-Gómez R, Campbell A, Dong JG, Yang SF, Gómez-Lim MA (1997) Ethylene biosynthesis in banana fruit: isolation of a genomic clone to ACC oxidase and expression studies. *Plant Sci* 123:123–131
- Lysák M, Doleelova M, Horry JP, Swennen R, Doleel J (1999) Flow cytometric analysis of nuclear DNA content in *Musa*. *Theor Appl Genet* 98:1344–1350
- Madhulatha P, Anbalagan M, Jayachandran S, Sakthivel N (2004) Influence of liquid pulse treatment with growth regulators on in vitro propagation of banana (*Musa* spp. AAA). *Plant Cell Tissue Organ Cult* 76:189–191
- Madhulatha P, Kirubakaran SI, Sakthivel N (2006) Effects of carbon sources and auxins on in vitro propagation of banana. *Biol Plant* 50:782–784
- Magee CJP (1939) Pathological changes in the phloem and neighbouring tissue of the banana (*Musa cavendishii* LAMB.) caused by the bunchy top virus. Department of Agriculture New South Wales, Science Bulletin 67
- Mainardi JA, Purgatto E, Vieira A Jr, Bastos WA, Cordenunsi BR, Nascimento JRO, Lajolo FM (2006) Effects of ethylene and 1-methylcyclopropene (1-MCP) on gene expression and activity profile of  $\alpha$ -1,4-glucan-phosphorylase during banana ripening. *J Agric Food Chem* 54:7294–7299
- Manrique-Trujillo SM, Ramírez-López AC, Ibarra-Laclette E, Gómez-Lim MA (2007) Identification of genes differentially expressed during ripening of banana. *J Plant Physiol* (in press)
- Mansoor S, Qazi S, Amin M, Khatri A, Khan IA, Raza S, Zafar Y, Briddon RW (2005) A PCR-based method, with internal control, for the detection of banana bunchy top virus in banana. *Mol Biotechnol* 30:167–169
- Marín-Rodríguez MC, Smith DL, Manning K, Orchard J, Seymour GB (2003) Pectate lyase gene expression and enzyme activity in ripening banana fruit. *Plant Mol Biol* 51:851–857
- Marroquin CG, Paduscheck C, Escalant JV, Teisson C (1993) Somatic embryogenesis and plant regeneration through cell suspension in *Musa acuminata*. *In Vitro Cell Dev Biol* 29P:43–46
- Martin KP, Pachathundikandi SK, Zhang CL, Slater A, Madassery J (2006) RAPD analysis of a variant of banana (*Musa* sp.) cv. Grande Naine and its propagation via shoot tip culture. *In Vitro Cell Dev Biol-Plant* 42:188–192
- Matsumoto K, Oka S (1998) Plant regeneration from protoplasts of Brazilian dessert banana (*Musa* spp., AAB group). *Acta Hort* 490:455–462
- Matsumoto K, Yamaguchi H (1990) Selection of aluminium-tolerant variants from irradiated protocorm-like bodies in banana. *Trop Agric* 67:229–232
- Matsumoto K, Vilarinhos AD, Oka S (2002) Somatic hybridization by electrofusion of banana protoplasts. *Euphytica* 125:317–324
- May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Bio/Technology* 13:486–492
- Medina-Suárez R, Manning K, Fletcher J, Aked J, Bird CR, Seymour GB (1997) Gene expression in the pulp of ripening bananas. *Plant Physiol* 115:453–461
- Morel G, Wetmore RH (1951) Fern callus tissue culture. *Am J Bot* 38:141–143
- Moriguchi T, Kita M, Hisada S, Endo-Inagaki T, Omura M (1998) Characterization of gene repertoires at mature stage of citrus fruits through random sequencing and analysis of redundant metallothionein-like genes expressed during fruit development. *Gene* 211:221–227
- Mota MV, Cordenunsi BR, Nascimento JRO, Purgatto E, Rosseto MRM, Lajolo FM (2002) Activity and expression of banana starch phosphatases during fruit development and ripening. *Planta* 216:325–333
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–493
- Nam YW, Tichit L, Leperlier M, Cuerq B, Marty I, Lelièvre JM (1999) Isolation and characterization of mRNAs differentially expressed during ripening of wild strawberry (*Fragaria vesca* L.) fruits. *Plant Mol Biol* 39:629–636
- Nascimento JRO, Cordenunsi BR, Lajolo FM, Alcocer MJC (1997) Banana sucrose-phosphate synthase gene expression during fruit ripening. *Planta* 203:283–288



- Nascimento JRO, Júnior AV, Bassinello PZ, Cordenunsi BR, Mainardi JA, Purgatto E, Lajolo FM (2006) Beta-amylase expression and starch degradation during banana ripening. *Postharvest Biol Technol* 40:41–47
- Navarro C, Escobedo RM, Mayo A (1997) In vitro plant regeneration from embryogenic cultures of a diploid and a triploid, Cavendish banana. *Plant Cell Tissue Organ Cult* 51:17–25
- Nguyen-Quoc B, N'Tchobo H, Foyer CH, Yelle S (1999) Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. *J Exp Bot* 50:785–791
- Novak FJ, Afza R, Van Duren M, Perea-Dallos M, Conger BV, Tang X (1989) Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp.). *Bio/Technology* 7:154–159
- Novak FJ, Afza R, Van Duren M, Omar MS (1990) Mutation induction by gamma irradiation of in vitro cultured shoot tips of banana and plantain (*Musa* cvs.). *Trop Agric* 67:21–28
- Okole BN, Schulz FA (1996) Micro-cross sections of banana and plantains (*Musa* spp.): morphogenesis and regeneration of callus and shoot buds. *Plant Sci* 116:185–195
- Onguso JM, Kahangi EM, Ndiritu DW, Mizutani F (2004) Genetic characterization of cultivated bananas and plantains in Kenya by RAPD markers. *Sci Hort* 99:9–20
- Ortiz R, Ferris RSB, Vuylsteke DR (1995) Banana, and plantain breeding. In: Gowen S (ed) *Bananas and plantains*. Chapman & Hall, London, pp 110–146
- Ortiz-Vázquez E, Kaemmer D, Zhang HB, Muth J, Rodríguez-Mendiola M, Arias-Castro C, James A (2005) Construction and characterization of a plant transformation-competent BIBAC library of the black Sigatoka-resistant banana *Musa acuminata* cv. Tuu Gia (AA). *Theor Appl Genet* 110:706–713
- Panis B, Van Wauwe A, Swennen R (1993) Plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa* spp.). *Plant Cell Rep* 12:403–407
- Panis B, Tette N, Van Nimmen K, Withers LA, Swennen R (1996) Cryopreservation of banana (*Musa* spp.) meristem cultures after preculture on sucrose. *Plant Sci* 21:95–106
- Panis B, Schoofs H, Thinh NT, Swennen R (2000) Cryopreservation of proliferating meristem cultures of banana. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm: current research progress and applications*. Proc JIRCAS/IPGRI Joint Int Workshop, IPGRI, Rome, pp 238–243
- Pathak N, Asif MH, Dhawan P, Srivastava MK, Nath P (2003) Expression and activities of ethylene biosynthesis enzymes during ripening of banana fruits and effect of 1-MCP treatment. *Plant Growth Regul* 40:11–19
- Payasi A, Misra PC, Sanwal GG (2004) Effect of phytohormones on pectate lyase activity in ripening *Musa acuminata*. *Plant Physiol Biochem* 42:861–865
- Pei XW, Chen SK, Wen RM, Ye S, Huang JQ, Zhang YQ, Wang BS, Wang ZX, Jia SR (2005) Creation of transgenic bananas expressing human lysozyme gene for Panama wilt resistance. *J Integrat Plant Biol* 47:971–977
- Pérez-Hernández JB, Swennen R, Sági L (2006) Number and accuracy of T-DNA insertions in transgenic banana (*Musa* spp.) plants characterized by an improved anchored PCR technique. *Transgenic Res* 15:139–150
- Peumans WJ, Barre A, Derycke V, Rougé P, Zhang W, May GD, Delcour JA, Van Leuven F, Van Damme EJM (2000) Purification, characterization and structural analysis of an abundant  $\beta$ -1,3-glucanase from banana fruit. *Eur J Biochem* 267:1188–1195
- Peumans WJ, Proost P, Swennen RL, Van Damme EJM (2002) The abundant class III chitinase homolog in young developing banana fruits behaves as a transient vegetative storage protein and most probably serves as an important supply of amino acids for the synthesis of ripening-associated proteins. *Plant Physiol* 130:1063–1072
- Pua EC, Lee YC (2003) Expression of a fruit-related cytochrome P450 cDNA in Cavendish banana (*Musa acuminata* cv. Williams). *Gene* 305:133–140
- Pua EC, Lim SSW, Liu P, Liu ZJ (2000) Expression of UDP-glucose pyrophosphorylase cDNA during fruit ripening of banana (*Musa acuminata*). *Aust J Plant Physiol* 27:1151–1159
- Pua EC, Ong CK, Liu P, Liu ZJ (2001) Isolation and expression of two pectate lyase genes during fruit ripening of banana (*Musa acuminata*). *Physiol Plant* 113:92–99

- Pua EC, Chandramouli P, Han P, Liu P (2003) Malate synthase gene expression during fruit ripening of banana (*Musa acuminata* cv. Williams). *J Exp Bot* 54:309–316
- Ray T, Dutta I, Saha P, Das S, Ray SC (2006) Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. *Plant Cell Tissue Organ Cult* 85:11–21
- Reid S, Ross GS (1997) Up-regulation of two cDNA clones encoding metallothionein-like proteins in apple fruit during cool storage. *Physiol Plant* 100:183–189
- Rethmeier N, Seurinck J, Van Montagu M, Cornelissen M (1997) Intron-mediated enhancement of transgene expression in maize is a nuclear, gene dependent process. *Plant J* 12:895–899
- Robinson JC (1995) Systems for cultivation and management. In: Gowen S (ed) Bananas and plantains. Chapman & Hall, London, pp 15–65
- Robinson JC, Fraser C, Eckstein K (1993) A field comparison of conventional suckers with tissue culture banana planting material over three crop cycles. *J Hortic Sci* 68:831–836
- Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal MJ, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. *Plant Cell Tissue Organ Cult* 82:57–66
- Sági L, Remy S, Panis B, Swennen R, Volckaert G (1994) Transient gene expression in electroporated banana (*Musa* spp. cv. 'Bluggoe', AAB group) protoplasts isolated from regenerable embryogenic cell suspensions. *Plant Cell Rep* 13:262–266
- Sági L, Panis B, Remy S, Schoofs H, De Smet K, Swennen R, Gammue BPA (1995) Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. *Bio/Technology* 13:481–485
- Sahijram L, Soneji JR, Bollamma KT (2003) Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro Cell Dev Biol-Plant* 39:551–556
- Santos CMR, Martins NF, Hörberg HM, de Almeida ERP, Coelho MCF, Togawa RC, da Silva FR, Caetano AR, Miller RNG, Souza Jr MT (2005) Analysis of expressed sequence tags from *Musa acuminata* ssp. *burmannicoides*, var. *Calcutta 4* (AA) leaves submitted to temperature stresses. *Theor Appl Genet* 110:1517–1522
- Schenk PM, Sági L, Remans T, Dietzgen RG, Bernard MJ, Graham MW, Manners JM (1999) A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. *Plant Mol Biol* 39:1221–1230
- Schenk PM, Remans T, Sági L, Elliott AR, Dietzgen RG, Swennen R, Ebert PR, Grof PL, Manners JM (2001) Promoters for pregenomic RNA of banana streak badnavirus are active for transgene expression in monocot and dicot plants. *Plant Mol Biol* 47:399–412
- Schenk RU, Hildebrandt HC (1972) Medium and techniques for induction and growth of monocotyledonous plant cell culture. *Can J Bot* 50:109–114
- Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold C, Tucker GA, Schuch W, Harding S, Grierson D (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant Mol Biol* 14:369–379
- Smith MK (1988) A review of factors influencing the genetic stability of micropropagated plants. *Fruits* 43:219–223
- Sreeramanan S, Maziah M, Rosli NM, Sariah M, Xavier R (2006) Enhanced tolerance against a fungal pathogen, *Fusarium oxysporum* f. sp. *cubense* (Race-1) in transgenic silk banana. *Int J Agric Res* 4:342–354
- Steffens JC, Harel E, Hunt MD (1994) Polyphenol oxidase. In: Ellis BE, Kuroki GW, Stafford HA (eds) Genetic engineering of plant secondary metabolism. Plenum, New York, pp 275–312
- Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R (2002) Practical considerations for pharmaceutical antibody production in different crop systems. *Mol Breed* 9:149–158
- Sunil Kumar GB, Ganapathi TR, Revathi CJ, Srinivas L, Bapat VA (2005) Expression of hepatitis B surface antigen in transgenic banana plants. *Planta* 222:484–493
- Thipyapong P, Melkonian J, Wolfe DW, Steffens JC (2004) Suppression of polyphenol oxidases increases stress tolerance in tomato. *Plant Sci* 167:693–703

- Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. *Plant Cell* 4:667–679
- Trainotti L, Zanin D, Casadoro G (2003) A cell wall-oriented genomic approach reveals a new and unexpected complexity of the softening in peaches. *J Exp Bot* 54:1821–1832
- Tripathi L, Tripathi JN, Hughes JD (2005) *Agrobacterium*-mediated transformation of plantain (*Musa* spp.) cultivar Agbagba. *Afr J Biotechnol* 4:1378–1383
- Trivedi PK, Nath P (2004) *MaExp1*, an ethylene-induced expansin from ripening banana fruit. *Plant Sci* 167:1351–1358
- Tucker G, Walley P, Seymour G (2006) Tomato. In: Pua EC, Davey MR (eds) *Biotechnology in agriculture and forestry*, vol 59. Transgenic crops IV. Springer, Berlin Heidelberg New York
- Turner WL, Plaxton WC (2001) Purification and characterization of banana fruit acid phosphatase. *Planta* 214:243–249
- Ude G, Pillay M, Nwakanma D, Tenkouano A (2002) Genetic diversity in *Musa acuminata* Colla and *Musa balbisiana* Colla and some of their natural hybrids using AFLP markers. *Theor Appl Genet* 104:1246–1252
- Vasil V, Clancy M, Murillo J, Rathus C, Nemes C, Finer JJ (1989) Increased gene expression by the first intron of maize *shrunk-1* locus in grass species. *Plant Physiol* 91:1575–1579
- Venkatachalam L, Thimmaraju R, Sreedhar RV, Bhagyalakshmi N (2006) Direct shoot and complete regeneration from leaf explants of 'silk' banana (AAB). *In Vitro Cell Dev Biol-Plant* 42:262–269
- Vuylsteke DR, Ortiz R (1996) Field performance of conventional vs. in vitro propagules of plantain (*Musa* spp., AAB group). *HortScience* 31:862–865
- Vuylsteke DR, Swennen RL, De Langhe EA (1991) Somaclonal variation in plantains (*Musa* spp., AAB group) derived from shoot-tip culture. *Fruits* 46:429–439
- Vuylsteke DR, Swennen RL, De Langhe EA (1996) Field performance of somaclonal variants of plantain (*Musa* ssp., AAB group). *J Am Soc Hortic Sci* 12:42–46
- Wang Y, Lu W, Jiang Y, Luo Y, Jiang W, Joyce D (2006) Expression of ethylene-related expansin genes in cool-stored ripening banana fruit. *Plant Sci* 170:962–967
- Whitehead AG (1998) *Plant nematode control*. CAB International, Wallingford, Oxon
- Wong C, Kiew R, Loh JP, Gan LH, Set O, Lee SK, Lum S, Gan YY (2001) Genetic diversity of the wild banana *Musa acuminata* Colla in Malaysia as evidenced by AFLP. *Ann Bot* 88:1017–1025
- Wong WC, Jalil M, Ong-Abdullah M, Othman RY, Khalid N (2006) Enhancement of banana plant regeneration by incorporating a liquid-based embryo development medium for embryogenic cell suspension. *J Hortic Sci Biotechnol* 81:385–390
- Wu YL, Yi GJ, Yang H, Zhou BR, Zeng JW (2005) Basal medium with modified nitrogen source and other factors influence the rooting of banana. *HortScience* 40:428–430
- Yang IC, Iommarini JP, Becker DK, Hafner GJ, Dale JL, Harding RM (2003) A promoter derived from taro bacilliform badnavirus drives strong expression in transgenic banana and tobacco plants. *Plant Cell Rep* 21:1199–1206
- Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. *Annu Rev Plant Physiol* 35:155–189
- Zhou J, Goldsbrough PB (1994) Functional homologs of fungal metallothionein genes from *Arabidopsis*. *Plant Cell* 6:875–884
- Zhuang JP, Su J, Chen WX (2006) Molecular cloning and characterization of fruit ripening related gene  $\beta$ -mannanase from banana fruit. *Agric Sci China* 5:277–283



## I.2 Citrus

L. PEÑA, M. CERVERA, C. FAGOAGA, J. ROMERO, J. JUÁREZ,  
J.A. PINA, and L. NAVARRO<sup>1</sup>

### 1 Introduction

The general area of origin of citrus is believed to be south-eastern Asia, though its cultivation probably started in China. Commercial citrus species and related genera belong to the order *Geraniales*, family *Rutaceae*, subfamily *Aurantoideae*. All rootstocks and varieties are included in the genus *Citrus*, except for kumquats (*Fortunella* spp.), and trifoliolate orange (*Poncirus trifoliata* L. Raf.), which is used exclusively as a rootstock. Commercial citrus fruits fall into several main groups, namely sweet oranges (*C. sinensis* (L.) Osb.), mandarins (*C. reticulata* Blanco, *C. deliciosa* Ten.), satsumas (*C. unshiu* (Mak.) Marc.), clementines (*C. clementina* Hort. ex Tan.), grapefruits (*C. paradisi* Macf.), pummelos (*C. grandis* (L.) Osb.), lemons (*C. limon* (L.) Burm. f.) and limes (*C. aurantifolia* (Christm.) Swing.). There are other species of relative importance in certain areas, such as sour oranges (*C. aurantium* L.), citrons (*C. medica* L.) and bergamots (*C. bergamia* Risso & Poit.). In addition, there are some commercial hybrids which are used as rootstocks including citranges (sweet orange  $\times$  trifoliolate orange) and citrumelos (grapefruit  $\times$  trifoliolate orange), and also important variety hybrids such as tangelos (mandarin  $\times$  grapefruit or pummelo), tangors (mandarin  $\times$  sweet orange) and mandarin hybrids.

### 2 The Importance of Citrus

Citrus is the most important fruit crop in the world, with more than 100 million tons produced from an area of 7.6 million hectares in 2005 (FAO 2006, <http://faostat.fao.org/>). It is grown in more than 100 countries all over the world, mainly in tropical and subtropical areas (at approximately 40° latitude each side of the equator), where favourable soil and climatic conditions prevail. Citrus fruits are marketed as mainly fresh fruit or as processed juice.

Citrus genotypes are grown commercially in a wide variety of soil and climatic conditions, as citrus trees are subjected to various abiotic and biotic stresses that may limit crop production. Citrus trees are also affected by many pests and diseases caused by nematodes, fungi, oomycetes, bacteria, spiroplas-

<sup>1</sup> Departamento Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain, e-mail: lpenya@ivia.es

mas, phytoplasmas, viruses and viroids. Disease is the main limitation on the development of the citrus industry in certain areas. This is the case with Huanglongbing, caused by the *Candidatus* bacterium *Liberobacter asiaticum*, found in most countries of south-east Asia, and more recently also in America (USA and Brazil). Other diseases such as citrus variegated chlorosis and citrus sudden death devastate millions of trees in Brazil. There are also diseases spread throughout the world, such as those produced by the oomycete *Phytophthora* sp. or by citrus tristeza virus (CTV), which preclude the use of certain excellent rootstocks, and severely restrict fruit production and the quality of important varieties in some countries. Efficient control of these diseases should be the main objective of citrus sanitation and improvement programs. Moreover, rootstocks are threatened by abiotic stresses such as acid, alkaline, and salty soils, flooding and drought, freezing and high temperatures. At the same time, the markets of developed countries demand fruits of increasing quality that are easy to peel, seedless, with attractive size and color, better organoleptic properties, ripening throughout the season, and with correct storage and shipment characteristics. In this situation, the genetic improvement of citrus is a major priority.

### 3 Genetic Improvement

Citrus species have a complex reproductive biology. Some important genotypes have total or partial pollen and/or ovule sterility and cannot be used as parents in breeding programs. There are also cases of cross- and self-incompatibility. Most species are partially apomictic, which means that adventitious embryos initiate directly from maternal nucellar cells, precluding the development of zygotic embryos, and thus the recovery of sexual progeny. The plant also possesses a relatively long juvenile growth phase and most members grown in subtropical areas require at least 5 years before flowering. These features, together with the large plant size of citrus, high heterozygosity and lack of knowledge of how the most important horticultural traits are inherited and of quantitative inheritance of most characteristics, have greatly impeded the genetic improvement of citrus through conventional breeding methods.

Breeding programs for citrus improvement began more than 100 years ago (Soost and Cameron 1975), but most rootstocks used today remain unchanged and the most important rootstock varieties originated from budspot mutations and chance seedlings. Only a few hybrid varieties are economically relevant in certain local markets. Perhaps the most significant achievements in the citrus improvement program are the production of Carrizo and Troyer citranges. These hybrids originated from a cross made in 1909 with the aim of introducing cold tolerance into edible fruits (Savage and Gardner 1965). To date, citranges have been widely used as rootstocks in Spain and the USA.

The development of genetic markers provides a new potential tool for citrus breeding. Linkage maps have been determined using isozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequence-characterized amplified regions (SCAR), amplified fragment length polymorphism (AFLP), microsatellites (single sequence repeats; SSRs) and cleaved amplified polymorphic sequences (CAPS). Although these studies have served to determine the mode of trait inheritance and can be useful for breeding purposes, map-based cloning of the corresponding genes remains unsolved. The only exception is the CTV-resistance gene from *Poncirus trifoliata*. Cloning of this gene is under way in several laboratories (Deng et al. 2001; Yang et al. 2003).

In most citrus countries, there has been increasing interest in tissue culture-derived biotechnologies for citrus sanitation and improvement. These include shoot tip grafting (Navarro et al. 1975; Navarro 1992), somatic hybridisation (Grosser et al. 2000), ploidy manipulation mainly for the production of triploid (seedless) varieties (Ollitrault et al. 1998) and genetic transformation.

## 4 Tissue Culture

Conventional methods to recover pathogen-free citrus plants, including the use of nucellar embryony and thermotherapy, have many limitations. Nucellar embryony is effective for elimination of virus and virus-like pathogens, but nucellar plants possess juvenile characteristics such as excessive growth vigor, thorn development and are late to bear fruit. These plants need to be grown for many years until the juvenile characters disappear before they can be acceptable for commercial propagation. In addition, nucellar-derived plants are not always true-to-type. Although thermotherapy results in true-to-type plants without juvenile characters, the technique is not effective for elimination of citrus viroids. Shoot tip grafting (STG) *in vitro* has been employed to eliminate citrus pathogens, including viroids. This technique refers to grafting a 0.1–0.2 mm shoot tip, composing the apical meristem plus two to three leaf primordia, excised from an infected plant onto a young rootstock seedling growing *in vitro* (Navarro et al. 1975; Navarro 1992). Apart from generation of pathogen-free citrus plants, STG also results in the production of true-to-type plants without juvenile characters. This technique has been widely used in most citrus sanitation programs worldwide. A quarantine tissue culture procedure has also been developed for safe introduction of citrus genotypes (Navarro 1992).

Production of triploid hybrids is currently the most promising approach to obtain seedless cultivars. Recovery of citrus sexual triploid hybrids ( $3x = 27$ ) has been reported since the early 1970s after  $2n \times 4n$ ,  $4n \times 2n$  and  $2n \times 2n$  crosses (Ollitrault et al. 1998). In the latter, the triploid embryos originated by fertilization of an unreduced diploid female gamete with a normal reduced haploid

male gamete. Seeds with triploid embryos are generally underdeveloped or aborted, and difficult to germinate. In addition, analysis of the ploidy level of large populations of citrus plants by cytological methods is very difficult. This may explain why triploids have not been widely used in citrus breeding. The development of methodologies for the culture of embryos and small seeds and for ploidy analysis by flow cytometry should allow more efficient production of citrus triploid sexual hybrids.

Somatic hybridization allows the production of somatic hybrids by incorporating the genomes of two parents without recombination, thus avoiding the problem of high heterozygosity in citrus. In citrus, this technology has been used extensively and its applications have been reviewed by Grosser et al. (2000).

## 5 Genetic Transformation

### 5.1 General Review

Genetic transformation may provide an efficient alternative for citrus improvement, opening the way for the introduction of specific traits into known genotypes without altering their elite genetic background. In the first attempt to transform citrus, protoplasts isolated from suspension-cultured cells of Trovita sweet orange were treated with a plasmid carrying a neomycin phosphotransferase (*nptII*) transgene in the presence of polyethylene glycol (PEG) (Kobayashi and Uchimiya 1989). Cell colonies proliferated in the selection medium containing 25 mg l<sup>-1</sup> kanamycin, but transgenic plants were not reported. Hidaka and Omura (1993) also reported transformation of Ohta ponkan protoplasts by electroporation, but only transformed cell colonies were obtained. In Page tangelo, Yao et al. (1996) generated 15 transformed embryo lines by transformation of embryogenic cells using particle bombardment. Again, transgenic plants were not obtained. Successful production of transgenic citrus plants was reported by Vardi et al. (1990), who introduced a plasmid harboring the *nptII* and chloramphenicol acetyltransferase (*cat*) transgenes into rough lemon (*C. jambhiri*) protoplasts in the presence of PEG. Transformed cells were grown into callus, from which transgenic plants were produced via somatic embryogenesis. Furthermore, production of transgenic citrus plants has also been achieved using *Agrobacterium tumefaciens*-mediated transformation. This was demonstrated by Hidaka et al. (1990) who co-cultivated suspension cells of several citrus species, including Washington navel and Trovita sweet oranges, Ohta ponkan and Kara mandarin, with *A. tumefaciens*. Transformed cells grown in the presence of kanamycin formed calli and somatic embryos, and a few transgenic plants of Washington navel orange were also obtained. Using a similar approach, two transgenic plantlets of Carrizo citrange were produced by co-cultivation of internodal stem segments

from in vitro-grown seedlings with *A. tumefaciens* harboring a binary plasmid with *nptII* and *uidA* ( $\beta$ -glucuronidase) marker transgenes (Moore et al. 1992). The same authors later reported a slight increase in transformation efficiency using a similar transformation protocol for regeneration of transgenic sour orange, lime and Carrizo citrange plants (Gutiérrez et al. 1997).

The first reliable protocol for production of citrus transgenic plants was reported by Kaneyoshi et al. (1994). In this study, the transformation frequency of more than 25% was obtained by co-cultivation of etiolated epicotyl segments of the citrus relative *Poncirus trifoliata* with *A. tumefaciens*. Later, this efficient transformation system also allowed the authors to transfer the human epidermal growth factor (*hEGF*) (Kobayashi et al. 1996) and the *rolC* gene from *Agrobacterium rhizogenes* (Kaneyoshi and Kobayashi 1999) into this species. In addition, a similar transformation procedure with modifications has been used to produce transgenic plants of Carrizo citrange (Peña et al. 1995a; LaMalfa et al. 2000; Wong et al. 2001; Yu et al. 2002), Washington navel orange (Bond and Roose 1998), Tarocco sweet orange (Gentile et al. 1998), Duncan grapefruit (Luth and Moore 2000), Rio Red grapefruit (Yang et al. 2000), Mexican lime (Koltunow et al. 2000), Xuegan sweet orange (Yu et al. 2002), Rangpur lime (*C. limonia* L. Osb.), Valencia and Natal sweet oranges (Almeida et al. 2003a; Boscariol et al. 2003) and *P. trifoliata* (Wong et al. 2001; Iwanami et al. 2004). Pérez-Molphe and Ochoa-Alejo (1998) also reported the production of transgenic lime by co-cultivation of internodal stem segments with *A. rhizogenes*. In Itaborai sweet orange, transgenic plants could be obtained via somatic embryogenesis by transformation of protoplasts in the presence of PEG (Fleming et al. 2000).

Transgenic plant production via somatic embryogenesis by protoplast transformation or co-cultivation of epicotyl segments from in vitro-grown seedlings with *A. tumefaciens* results in plants with a long juvenile growth phase. It is therefore imperative to develop the transformation systems for mature plants of citrus in order to overcome juvenility. The first step in such a direction was the use of glasshouse-grown plants as source material. Peña et al. (1995b) reported the regeneration of transgenic Pineapple sweet orange by co-cultivation of internodal stem segments (1 cm in length) from stem pieces (20 cm in length) from 6- to 12-month-old glasshouse-grown seedlings with *A. tumefaciens*. However, the use of internodal stem segments from mature citrus trees was shown to possess low regenerative potential (Cervera et al. 1998a). This problem appears to be overcome by grafting the buds from adult trees onto vigorously growing seedlings. A comparative study on regeneration from stem segments from the first, second and third flushes of newly grafted invigorated mature sweet orange plants showed that explants from the first and second flushes produced similar regeneration frequencies, which was significantly higher than that of the explants from the third flush. The first flush of the adult plants was therefore selected as source tissue for genetic transformation study; *A. tumefaciens* strain EHA105 derived from the succinamopine strain A281 was also chosen because it was demonstrated to induce super-transformation

in citrus (Cervera et al. 1998c). Results showed that transgenic Pineapple sweet orange flowered and set fruits after 12–15 months of growth in the glasshouse, whereas non-transformed controls required at least 8 years of growth before flowering (Cervera et al. 1998a). This transformation system has been later extended to other citrus genotypes, including other sweet oranges, Mexican lime, sour orange, alemow, lemon and Cleopatra mandarin (Peña et al. 1997; Domínguez et al. 2000; Ghorbel et al. 2000, 2001a; Almeida et al. 2003b; our unpublished results).

More recently, other factors have been shown to affect citrus transformation. These factors include the use of appropriate marker genes (Ghorbel et al. 1999), transformation vectors (Ghorbel et al. 2001a), co-cultivation conditions, culture media, and determination of competent cells for transformation in citrus explants (Peña et al. 2004), and adequate selection conditions (Domínguez et al. 2004).

In general, rooting of transgenic shoots in citrus, except for *P. trifoliata* and grapefruit, is rather inefficient and limits the recovery of whole transgenic plants. This problem is alleviated by the report by Peña et al. (1995a), who proposed the use of shoot tip grafting as an alternative to rooting for generating transgenic shoots. A frequency of 100% successful grafts was obtained by using transgenic shoot apical ends (0.1–0.5 cm in height) as scions. Shoot tip grafting has been demonstrated to be reliable and widely applicable (Peña et al. 1995b, 1997; Kobayashi et al. 1996; Cervera et al. 1998a, b; Bond and Roose 1998; Gentile et al. 1998; Ghorbel et al. 2000, 2001a; Domínguez et al. 2000; LaMalfa et al. 2000; Wong et al. 2001; Yu et al. 2002; Almeida et al. 2003a, b; Boscardiol et al. 2003; Iwanami et al. 2004).

## 5.2 Transformation Protocols

Aseptically germinated 4- to 5-week-old seedlings are used as the starting material for genetic transformation of *P. trifoliata* and Carrizo citrange. In order to initiate plant cultures, stored seeds are peeled, and seed coats are removed, disinfected for 10 min, in a 0.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20, and rinsed three times with sterile distilled water. These seedlings are grown in MS salts solidified with 10 g l<sup>-1</sup> agar, with the medium pH of 5.7. The cultures are kept at 26 °C in the dark for the first 2 weeks, followed by 3 weeks under a 16-h photoperiod, with a light intensity of 45  $\mu\text{E m}^{-2}\text{s}^{-1}$ . Older tissues, such as shoots from 4- to 12-month-old glasshouse-grown vigorous seedlings, are also used as the source of tissue for transformation of sweet orange, sour orange, lime, alemow, lemon and mandarin. For transformation of mature tissues, new shoots elongated from buds, collected from trees maintained in a screenhouse (pathogen-free Germplasm Bank Collection of the IVIA), grafted onto seedlings of a vigorous rootstock grown under glasshouse conditions (18–27 °C), are used as starting material. Stem pieces (20 cm in length) are stripped of their leaves and thorns, disin-



fectected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile distilled water.

*Agrobacterium tumefaciens* strain EHA105 carrying a binary plasmid is used for transformation. Bacteria are cultured overnight in an orbital shaker at 28 °C and 200 rpm in LB (Luria Bertani) medium containing the appropriate antibiotics. After the bacterial cells are pelleted at 3,500 rpm for 10 min, they are resuspended and diluted to  $4 \times 10^7$  cells  $\text{ml}^{-1}$  in liquid medium, consisting of MS salts solution, 0.2  $\text{mg l}^{-1}$  thiamine HCl, 1  $\text{mg l}^{-1}$  pyridoxine HCl, 1  $\text{mg l}^{-1}$  nicotinic acid and 3% (w/v) sucrose. Epicotyl or internodal stem segments (1 cm long) are cut transversely and incubated for 15 min in 15 ml of bacterial suspension by gentle shaking. The infected explants are blotted dry on sterile filter paper and co-cultivated for 3 days on CM medium consisting of MS salts, 1  $\text{mg l}^{-1}$  thiamine HCl, 1  $\text{mg l}^{-1}$  pyridoxine HCl, 1  $\text{mg l}^{-1}$  nicotinic acid, 3% (w/v) sucrose, 2  $\text{mg l}^{-1}$  indole-3-acetic acid, 1  $\text{mg l}^{-1}$  2-isopentenyl-adenine, 2  $\text{mg l}^{-1}$  2,4-dichlorophenoxyacetic acid and 0.8% (w/v) agar. Co-cultivation in the medium rich in auxins is believed to facilitate plant transformation by inducing the cells at the cut ends of the explants to undergo dedifferentiation, cell division and callus proliferation (Peña et al. 1997; Cervera et al. 1998a; Domínguez et al. 2004; Peña et al. 2004).

After co-cultivation, explants are blotted dry with sterile filter paper and transferred to SRM medium consisting of MS salts, 0.2  $\text{mg l}^{-1}$  thiamine HCl, 1  $\text{mg l}^{-1}$  pyridoxine HCl, 1  $\text{mg l}^{-1}$  nicotinic acid, 3% sucrose, 1% agar and 100  $\text{mg l}^{-1}$  kanamycin for the selection of transgenic shoots. The SRM also contains 250  $\text{mg l}^{-1}$  vancomycin and 500  $\text{mg l}^{-1}$  cefotaxime to control bacterial over-growth. This medium is supplemented with 3  $\text{mg l}^{-1}$  benzylaminopurine (BAP) for sweet orange and citrange, 1  $\text{mg l}^{-1}$  BAP for lime, lemon, alemow and mandarin, and 1  $\text{mg l}^{-1}$  BAP and 0.3  $\text{mg l}^{-1}$  naphthaleneacetic acid (NAA) for sour orange. Cultures are grown in the dark for 4 weeks at 26 °C and then maintained under a 16-h photoperiod with the light intensity of  $45 \mu\text{E m}^{-2}\text{s}^{-1}$ . It was observed that explants cultured in the dark favored callus formation and transgenic shoot regeneration (Peña et al. 1997; Cervera et al. 1998a). Meanwhile, it also avoided regeneration of escape shoots that could be stimulated by the exposure of explants directly to the light. For sour orange, it was speculated that the combination BAP/NAA in the SRM medium was more favorable than BAP alone in stimulating divisions and differentiation from the transformed cells (Ghorbel et al. 2000; Peña et al. 2004). Shoots usually develop from the cut ends 3–5 weeks after co-cultivation. Their transgenic nature is verified by histochemical assay for GUS activity (Peña et al. 1995a), or GFP expression (Ghorbel et al. 1999). The apical portions of transgenic shoots are then grafted in vitro onto seedling of the rootstock Troyer citrange (Peña et al. 1995a).

For rootstock preparation, Troyer citrange seeds are peeled to remove both seed coats, disinfected for 10 min in 0.5% sodium hypochlorite solution containing 0.1% Tween-20, rinsed three times with sterile water, and germinated on semi-solid hormone-free MS medium. Seeds are sown individually in tubes and grown in the dark at 27 °C. After 2 weeks, the seedlings are decapitated

to leave 1–1.5 cm of the epicotyls. The roots are reduced to 4–6 cm and the cotyledons, together with the axillary buds, are removed. The regenerated shoot apical ends are then placed in contact with the vascular ring on the cut surfaces of the decapitated seedling epicotyls. Alternatively, shoot apices, when larger than 0.4 cm, can be inserted into a lateral incision or in a vertical incision along the length of the epicotyl, starting at the point of decapitation (Peña et al. 1995a, b). Grafted plants are cultured in a liquid medium composed of MS salts, 100 mg l<sup>-1</sup> m-inositol, 0.2 mg l<sup>-1</sup> thiamine HCl, 1 mg l<sup>-1</sup> pyridoxine HCl, 1 mg l<sup>-1</sup> nicotinic acid and 75 g l<sup>-1</sup> sucrose. The cultures are kept under a 16-h photoperiod with a light intensity of 45  $\mu\text{E m}^{-2}\text{s}^{-1}$  at 25 °C. Scions develop two to four expanded leaves 3–4 weeks after grafting. New grafting of the in vitro-growing plants on vigorous rootstocks in the glasshouse allows the rapid acclimatization and development of the plants.

## 6 Citrus Improvement by Genetic Engineering

Although there are many reports on the introduction of transgenes of interest into citrus, only a few of them describe how the expression of the transgene affects the phenotype of the modified rootstock or variety. This is due, in part, to the fact that in most cases juvenile tissues are transformed and consequently the fruit cannot be evaluated. Furthermore, it is also due to the difficulties of performing challenge assays against pathogens with woody plants. Early attempts to transform citrus only involved the marker genes such as *nptII* and *uidA* for optimization of the transformation system. This also led to efficient transfer of the *hEGF* (Kobayashi et al. 1996) and *rolC* (Kaneyoshi and Kobayashi 1999) genes into *P. trifoliata*. As the precise role of EGF in vivo was unknown, the first work only served to demonstrate that it was possible to express human bioactive peptides in transgenic trees.

### 6.1 Tree Performance and Fruit Quality

It was observed that most transgenic citrus expressing *rolC* were dwarfed (Kaneyoshi and Kobayashi 1999). The degree of dwarfing differed amongst the lines, most of which showed less than half the size of the control. This character may be important for the production of dwarf rootstocks, although there is no report on the use of these transgenic plants for this purpose. Gentile et al. (1998) reported regeneration of transgenic Tarocco orange plants expressing the *rol* genes, but their phenotypes were not characterized. Apart from dwarfism, modifications of other plant characters have also been explored. Li et al. (2002), in an attempt to produce male sterile transformants, reported the generation of Ponkan transgenic plants expressing a ribonuclease gene under the control of a tapetum-specific promoter. However, as transgenic plants remained at the juvenile stage, several years of cultivation are needed before male sterility can



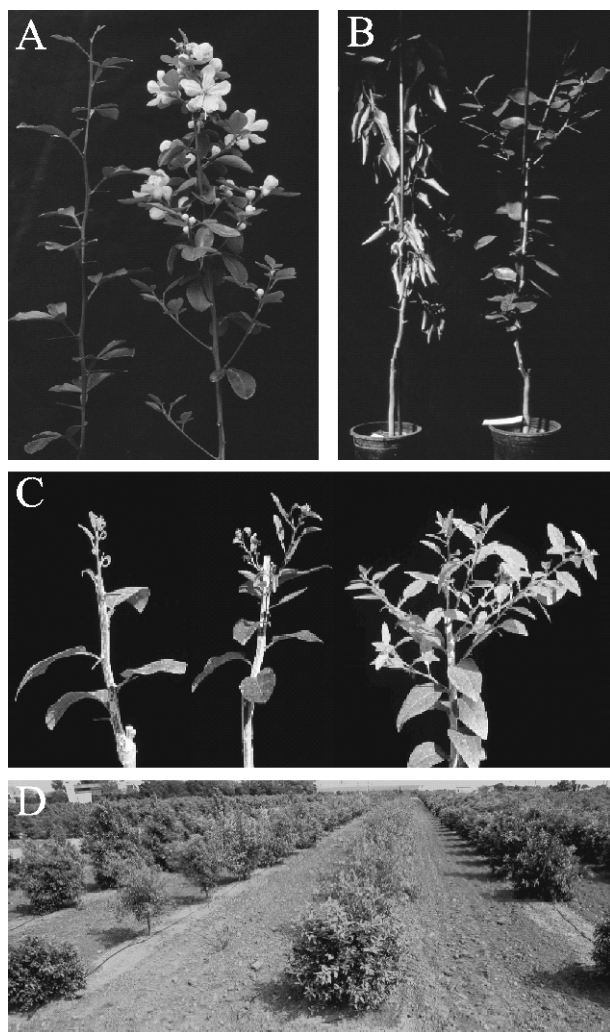
be evaluated. A similar scenario has also been observed in another study, where juvenile transgenic limes expressed genes for decreased seed set (Koltunow et al. 2000). Juvenile Duncan grapefruit expressing the carotenoid biosynthetic genes were also generated, but there was no apparent difference in color in vegetative tissues between transgenic and control plants (Costa et al. 2002).

## 6.2 Shortening the Juvenile Growth Phase

The long juvenile growth phase has been one of the major factors that has impeded the program for genetic improvement of citrus as it prolongs the time required for analysis of late traits, such as fruit features. With the aim of accelerating flowering time, we have transformed juvenile Carrizo citrange seedlings that constitutively expressed the *Arabidopsis* *LEAFY* or *APETALA1* genes, which were shown to promote flower initiation in *Arabidopsis* (Mandel and Yanofsky 1995; Weigel and Nilsson 1995). Both types of transgenic citrus plants produced fertile flowers and fruits as early as the first year, notably through a mechanism involving a dramatic shortening of their juvenile phase (Fig. 1A). Furthermore, expression of *APETALA1*, being as efficient as *LEAFY* in flower initiation, did not result in severe developmental abnormality. Both types of transgenic trees flowered again in consecutive years and the flowering response was shown to be under environmental control. In addition, sexual and nucellar-derived transgenic seedlings possessed a very short juvenile phase and flowered in their first spring, demonstrating the stability and inheritance of this trait (Peña et al. 2001). These results indicate the feasibility of using independent *APETALA1* transgenic plants as parents in crosses with non-transformed genotypes to generate 50% of the progeny flowering and setting fruits within 1–2 years and, as a result, allowing the plants to be evaluated earlier for fruit features. By re-transformation of *APETALA1* transgenic citrus plants, it is also possible to rapidly test the expression of certain transgenes under the control of flower organ- or fruit-specific promoters as a system to develop seedless varieties, modify fruit color and aroma, or favor easy-peeling characteristics.

## 6.3 Abiotic Stress Tolerance

Salinity is a major limiting factor for citrus production in many areas worldwide. Carrizo citrange has been considered an excellent citrus rootstock, but it is highly sensitive to salt stress. We have previously generated transgenic Carrizo citrange expressing the yeast gene *HAL2* (Cervera et al. 2000b), which is implicated in salt tolerance mechanisms (Murguía et al. 1995). Plants that showed high levels of *HAL2* transcript were selected for salt tolerance evaluation, but their salt tolerance/sensitivity was shown to be comparable to that of control plants. Results of another transgenic study indicate that chilling tolerance is associated with ethylene, as demonstrated in transgenic *P. trifoliata*



**Fig. 1.** Incorporation of genes of potential agricultural interest into citrus. **A** Over-expression of *APETALA1* (*API*) gene from *Arabidopsis* in Carrizo citrange transgenic plants induces early flowering and greatly reduces the juvenile period. Transgenic *API* plant flowering 13 months after its transfer to the glasshouse (right) compared to a control plant (left). **B** Resistance against *Phytophthora citrophthora* in transgenic sweet orange plants expressing the pathogenesis-related osmotin-like protein P23 from tomato. Comparison between a plant from a highly resistant line (right) and a control plant (left). **C** Engineering citrus resistance to CTV. A non-transgenic control lime (left), transgenic *p25* CP lime showing moderate protection (centre) and transgenic *p25* CP lime showing resistance against CTV infection (right). **D** Release of genetically modified citrus plants under controlled field conditions at the IVIA

and Carrizo citrange plants expressed an antisense 1-aminocyclopropane-1-carboxylate (ACC) synthase gene (*CS-ACS1*) of *C. sinensis* (Wong et al. 2001). In these transgenic plants, the level of endogenous ACC was partially repressed in response to chilling treatment. It was speculated that the reduced concentration of ACC in transgenic citrus tissues might be useful to reduce the damages caused by chilling injury in the fruits.

#### 6.4 Biotic Stress Resistance

*Phytophthora citrophthora* is the most widely spread oomycete in citrus growing areas and represents one of the major causes of crop losses. Constitutive over-expression of genes for proteins involved in the plant defense mechanism against diseases is one of the strategies proposed to increase resistance of plants to fungal pathogens. A 23-kDa pathogenesis-related protein, P23, which was induced in tomato in response to infection of citrus exocortis viroid, possessed antifungal activity in *in vitro* assays. We have produced transgenic Pineapple sweet orange over-expressing the *P23* gene. Several transgenic plants were challenged with *P. citrophthora* by inoculating whole plants with fungal cultures, and at least one of the lines displayed a higher survival rate than the control (Fig. 1B). These results provide evidence of the antifungal activity of the P23 protein against *P. citrophthora*, suggesting that this may be employed as a strategy aimed at the engineering of *Phytophthora* disease resistance in citrus (Fagoaga et al. 2001).

CTV is the causal agent of the most important virus disease of citrus in the world. Several strategies have been employed to engineer plant resistance to viral pathogens. Most are based on the concept of pathogen-derived resistance, which proposes that the introduction and expression in plants of viral sequences can interfere with the life cycle of the same or a closely related challenging virus, thus providing resistance to infection. Gutierrez et al. (1997) reported the production of a few transgenic plants of sour orange, Mexican lime and Carrizo citrange with the *p25* gene from CTV, but challenge analysis of these plants has not been reported to date. Several transgenic Rio Red grapefruit plants expressing an untranslatable version of the *p25* coat protein gene from CTV and the *Galanthus nivalis* agglutinin gene have also been generated (Yang et al. 2000). These transgenic plants were either transferred to the field for aphid-challenge or graft-inoculated under controlled glasshouse conditions, but only moderate protection was observed (Mirkov, personal communication). Over-expression of translatable and non-translatable versions of the coat protein *p25* gene, the *RdRp* gene and the 3' end of the untranslatable region did not confer disease resistance in transgenic Duncan grapefruit (Febres et al. 2003).

We also introduced the coding and non-coding versions of the coat protein *p25* gene into Mexican lime, which is highly sensitive to CTV, to evaluate pathogen-derived resistance in these plants (Domínguez et al. 2000, 2002a,b,c).

When plants propagated from each *p25* transgenic line were graft- or aphid-inoculated with CTV, two types of response to viral challenge were observed. There were lines that showed that CTV symptoms developed similar to those of non-transgenic controls, whereas other lines exhibited protection against the virus (Fig. 1C). This protection was characterised by 10–33% of transgenic propagations that were resistant to CTV, while the remaining propagations showed a significant delay in virus accumulation and symptom onset (Domínguez et al. 2002a). This is the first report demonstrating pathogen-derived resistance in transgenic plants against a *Closterovirus* member in its natural host. However, transgenic lines expressing different untranslatable versions of the *p25* gene did not show protection against CTV challenge (Domínguez et al. 2002b, c). In another study, transgenic sour orange plants that expressed and did not express the *p25* gene were also generated (Ghorbel et al. 2000), but no viral challenge was performed. A recent study showed that transgenic *P. trifoliata* plants that expressed the coat protein gene from *Citrus mosaic virus* (CiMV) were more tolerant to virus (Iwanami et al. 2004). After the transgenic plants were subject to virus challenge, one of the lines was shown to exhibit high tolerance with only 7.1% infection, compared to 65.1% infection in non-transformed control plants. Other transgenic plants showed differential responses ranging from susceptibility to moderate tolerance to the virus.

The 3'-terminal gene of CTV codes for a *p23* protein, which is an RNA-binding protein that possesses a motif rich in cysteine and histidine residues. For this reason, *p23* has been considered to play a regulatory role in CTV replication or gene expression. To investigate whether over-expression of this protein in transgenic plants could affect the normal CTV infectious process, we produced transgenic lime, sour orange, sweet orange and *P. trifoliata* plants expressing the full length or a truncated version of *p23*. Constitutive expression of *p23* was shown to induce phenotypic aberrations resembling symptoms incited by CTV in non-transformed control citrus plants, whereas transgenic plants that expressed the truncated *p23* were normal. The onset of CTV-like symptoms in *p23*-transgenic plants was associated with the expression of *p23*, whose level was paralleled to the symptom intensity (Ghorbel et al. 2001b; Fagoaga et al. 2005). These results demonstrate that *p23* is involved in symptom development and that it is likely to play a key role in CTV pathogenesis.

## 6.5 Field Trial

A release of genetically modified citrus plants under controlled field conditions was initiated in 1997 (Fig. 1D). The release site is located at the IVIA in an experimental field with an area of 1,638 m<sup>2</sup>. There are 130 trees, including 16 transgenic plants of Pineapple sweet orange, 16 transgenic plants of lime and 16 transgenic plants of Carrizo citrange (two plants from eight independent transgenic lines for each case) (Cervera et al. 2000a). In addition, there are eight non-transgenic control plants from each of the species and an external border of

58 non-transgenic trees of *Clemenules clementine*. The purpose of the release is to investigate morphological and phenological characteristics of the transgenic trees, expression of the transgenes in leaves, flowers and fruits, stability of the transgenes, transmission of the transgenes to the progeny, and the frequency of transgene dispersal through the pollen to non-transgenic monoembryonic citrus trees (*Clemenules clementine*). The trial has been approved by the Spanish Ministry of Environment (permit no. B/ES/96/15) and is in accordance with Article 9 of Directive 90/220 of the European Union. Until now, transgenic plants are morphologically and phenologically normal, as are non-transgenic controls, and transgenes are stably expressed over different seasons and in different plant tissues and organs.

## 7 Conclusions

Currently, many citrus species can be readily transformed with the correct selection of vectors, the use of vigorous source material competent for transformation and regeneration, and the establishment of adequate inoculation, co-cultivation, regeneration-selection culture media and conditions, and the recovery of transgenic shoots through micrografting. This has permitted the introduction of transgenes into citrus with the aim of improving rootstock and variety performance. However, efforts need to be made to increase the transformation frequency and to develop efficient transformation systems for other economically important citrus genotypes such as mandarins, including clementines and satsumas. An efficient transformation system is also an important tool for functional genomic analyses, leading to identification of citrus genes for the desirable traits for improvement of fruit quality. In addition, future efforts must also focus on studying how the modified genome is affected over years by the insertion of a foreign gene and its expression, and on the potential environmental impacts of releasing transgenic citrus plants into the field.

**Acknowledgements.** The authors wish to thank J.E. Peris and A. Navarro for their excellent technical assistance. This research was supported by INIA and CICYT grants, and currently by grants AGL2003-01644 and AGL2006-03673 from CICYT.

## References

- Almeida WAB, Mourao Filho FAA, Mendes BMJ, Pavan A, Rodríguez APM (2003a) *Agrobacterium*-mediated transformation of *Citrus sinensis* and *Citrus limonia* epicotyl segments. *Sci Agric* 60:23–29
- Almeida WAB, Mourao Filho FAA, Pino LE, Boscariol RL, Rodríguez APM, Mendes BMJ (2003b) Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. *Plant Sci* 164:203–211

- Bond JE, Roose ML (1998) *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. *Plant Cell Rep* 18:229–234
- Boscariol RL, Almeida WAB, Derbyshire MTVC, Mourao Filho FAA, Mendes BMJ (2003) The use of PMI/mannose selection system to recover transgenic sweet orange plants (*Citrus sinensis* L. Osbeck). *Plant Cell Rep* 16:271–278
- Cervera M, Pina JA, Juárez J, Navarro L, Peña L (1998a) *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. *Plant Cell Rep* 18:271–278
- Cervera M, Juárez J, Navarro A, Pina JA, Durán-Vila N, Navarro L, Peña L (1998b) Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. *Transgenic Res* 7:51–59
- Cervera M, López MM, Navarro L, Peña L (1998c) Virulence and supervirulence of *Agrobacterium tumefaciens* in woody fruit plants. *Physiol Mol Plant Pathol* 52:67–78
- Cervera M, Pina JA, Juárez J, Navarro L, Peña L (2000a) A broad exploration of a transgenic population of citrus: stability on gene expression and phenotype. *Theor Appl Genet* 100:670–677
- Cervera M, Ortega C, Navarro A, Navarro L, Peña L (2000b) Generation of transgenic citrus plants with the tolerance-to-salinity gene *HAL2* from yeast. *J Hortic Sci Biotechnol* 75:26–30
- Costa MGC, Otoni WC, Moore GA (2002) An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Rep* 21:365–373
- Deng Z, Huang S, Ling P, Yu C, Tao Q, Chen C, Wendell MK, Zhang H-B, Gmitter FG Jr (2001) Fine genetic mapping and BAC contig development for the citrus tristeza virus resistance gene locus in *Poncirus trifoliata* (Raf.). *Mol Genet Genomics* 265:739–747
- Domínguez A, Guerri J, Cambra M, Navarro L, Moreno P, Peña L (2000) Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep* 19:427–433
- Domínguez A, Hermoso de Mendoza A, Guerri J, Cambra M, Navarro L, Moreno P, Peña L (2002a) Pathogen-derived resistance to *Citrus tristeza virus* (CTV) in transgenic Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) plants expressing its *p25* coat protein gene. *Mol Breed* 10:1–10
- Domínguez A, Fagoaga C, Navarro L, Moreno P, Peña L (2002b) Regeneration of transgenic citrus plants under non selective conditions results in high frequency recovery of plants with silenced transgenes. *Mol Genet Genomics* 267:544–556
- Domínguez A, Fagoaga C, Navarro L, Moreno P, Peña L (2002c) Constitutive expression of untranslatable versions of the *p25* coat protein gene in Mexican lime (*Citrus aurantifolia* (Christm.) Swing.) transgenic plants does not confer resistance to *Citrus tristeza virus* (CTV). In: Durán-Vila N, Milne RG, DaGraca JV (eds) *Proceedings of the 15th Annual Conference of the International Organization of Citrus Virologists*, University of California, Riverside, California, pp 341–344
- Domínguez A, Cervera M, Pérez R, Romero J, Fagoaga C, Cubero J, López MM, Juárez J, Navarro L, Peña L (2004) Characterisation of regenerants obtained under selective conditions after *Agrobacterium*-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies. *Mol Breed* 14:171–183
- Fagoaga C, Rodrigo I, Conejero V, Hinarejos C, Tuset JJ, Arnau J, Pina JA, Navarro L, Peña L (2001) Increased tolerance to *Phytophthora citrophthora* in transgenic orange plants overexpressing a tomato pathogenesis related protein PR-5. *Mol Breed* 7:175–181
- Fagoaga C, López C, Moreno P, Navarro L, Flores R, Peña, L (2005) Viral-like leaf symptoms induced by the ectopic expression of the *p23* gene of citrus tristeza virus are specific of citrus species and their intensity does not depend on the pathogenicity of the virus strain. *Mol Plant-Microbe Interact* 18:435–445
- Febres VJ, Niblett CL, Lee RF, Moore GA (2003) Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with citrus tristeza closterovirus genes. *Plant Cell Rep* 21:421–428
- Fleming GH, Olivares-Fuster O, Fatta Del-Bosco S, Grosser JW (2000) An alternative method for the genetic transformation of sweet orange. *In Vitro Cell Dev Biol-Plant* 36:450–455



- Gentile A, LaMalfa S, Deng ZN, Domina F, Nicolosi E, Tribulato E (1998) Transgenic citrus: first experiences with Rol genes. *Riv Fruttic Ortofloricolt* 61:59–61
- Ghorbel R, Juárez J, Navarro L, Peña L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. *Theor Appl Genet* 99:350–358
- Ghorbel R, Domínguez A, Navarro L, Peña L (2000) High efficiency genetic transformation of sour orange (*Citrus aurantium* L.) and production of transgenic trees containing the coat protein gene of *Citrus tristeza virus*. *Tree Physiol* 20:1183–1189
- Ghorbel R, LaMalfa S, López MM, Petit A, Navarro L, Peña L (2001a) Additional copies of *virG* from pTiBo542 provide a super-transformation ability to *Agrobacterium tumefaciens* in citrus. *Physiol Mol Plant Pathol* 58:103–110
- Ghorbel R, López C, Moreno P, Navarro L, Flores R, Peña L (2001b) Transgenic citrus plants expressing the citrus tristeza virus p23 protein exhibit viral-like symptoms. *Mol Plant Pathol* 2:27–36
- Grosser JW, Ollitrault P, Olivares-Fuster O (2000) Somatic hybridization in *Citrus*: an effective tool to facilitate variety improvement. *In Vitro Cell Dev Biol-Plant* 36:439–449
- Gutiérrez MA, Luth DE, Moore GA (1997) Factors affecting *Agrobacterium*-mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep* 16:745–753
- Hidaka T, Omura M (1993) Transformation of citrus protoplasts by electroporation. *J Jpn Soc Hortic Sci* 62:371–376
- Hidaka T, Omura M, Ugaki M, Tomiyama M, Kato A, Ohshima M, Motoyoshi F (1990) *Agrobacterium*-mediated transformation and regeneration of *Citrus* spp. from suspension cells. *Jpn J Breed* 40:199–207
- Iwanami T, Shimizu T, Ito T, Hirabayashi T (2004) Tolerance to *Citrus mosaic virus* in transgenic trifoliolate orange lines harboring capsid polyprotein gene. *Plant Dis* 88:865–868
- Kaneyoshi J, Kobayashi S (1999) Characteristics of transgenic trifoliolate orange (*Poncirus trifoliata* Raf.) possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. *J Jpn Soc Hortic Sci* 68:734–738
- Kaneyoshi J, Kobayashi S, Nakamura Y, Shigemoto N, Doi Y (1994) A simple and efficient gene transfer system of trifoliolate orange. *Plant Cell Rep* 13:541–545
- Kobayashi S, Uchimiya H (1989) Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer. *Jpn J Genet* 64:91–97
- Kobayashi S, Nakamura Y, Kaneyoshi J, Higo H, Higo K (1996) Transformation of kiwifruit (*Actinidia chinensis*) and trifoliolate orange (*Poncirus trifoliata*) with a synthetic gene encoding the human epidermal growth factor (*hEGF*). *J Jpn Soc Hortic Sci* 64:763–769
- Koltunow AM, Brennan P, Protosaltis S, Nito N (2000) Regeneration of West Indian limes (*Citrus aurantifolia*) containing genes for decreased seed set. *Acta Hort* 535:81–91
- LaMalfa S, Gentile A, Deng ZN, Domina F (2000) Citrus genetic transformation with a vital reporter gene: expression of green fluorescent protein in Troyer citrange. *Italus Hortus* 7:17–21
- Li DD, Shi W, Deng XX (2002) *Agrobacterium*-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene. *Plant Cell Rep* 21:153–156
- Luth D, Moore G (2000) Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Tissue Organ Cult* 57:219–222
- Mandel MJ, Yanofsky MF (1995) A gene triggering flower formation in *Arabidopsis*. *Nature* 377:522–524
- Moore GA, Jacono CC, Neidigh JL, Lawrence SD, Cline K (1992) *Agrobacterium*-mediated transformation of citrus stem segments and regeneration of transgenic plants. *Plant Cell Rep* 11:238–242
- Murguía JR, Bellés JM, Serrano R (1995) A salt-sensitive 3'(2'), 5'-bisphosphate nucleotidase involved in sulfate activation. *Science* 267:232–234

- Navarro L (1992) Citrus shoot tip grafting *in vitro*. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 18. High-tech and micropropagation II. Springer, Berlin Heidelberg New York, pp 328–338
- Navarro L, Roistacher CN, Murashige T (1975) Improvement of shoot-tip grafting in vitro for virus-free citrus. *J Am Soc Hortic Sci* 100:471–479
- Ollitrault P, Dambier D, Sudahono, Mademba-Sy F, Vanel F, Luro F, Aubert B (1998) Biotechnology for triploid mandarin breeding. *Fruits* 53:307–317
- Peña L, Cervera M, Juárez J, Ortega C, Pina JA, Durán-Vila N, Navarro L (1995a) High efficiency *Agrobacterium*-mediated transformation and regeneration of citrus. *Plant Sci* 104:183–191
- Peña L, Cervera M, Juárez J, Navarro A, Pina JA, Durán-Vila N, Navarro L (1995b) *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. *Plant Cell Rep* 14:616–619
- Peña L, Cervera M, Juárez J, Navarro A, Pina JA, Navarro L (1997) Genetic transformation of lime (*Citrus aurantifolia* Swing.): factors affecting transformation and regeneration. *Plant Cell Rep* 16:731–737
- Peña L, Martín-Trillo M, Juárez J, Pina JA, Navarro L, Martínez-Zapater JM (2001) Constitutive expression of *Arabidopsis* *LEAFY* and *APETALA1* genes in citrus reduces their generation time. *Nat Biotechnol* 19:263–267
- Peña L, Pérez R, Cervera M, Juárez JA, Navarro L (2004) Early events in *Agrobacterium*-mediated genetic transformation of citrus explants. *Ann Bot* 94:67–74
- Pérez-Molphe E, Ochoa-Alejo N (1998) Regeneration of transgenic plants of Mexican lime from *Agrobacterium rhizogenes*-transformed tissues. *Plant Cell Rep* 17:591–596
- Savage EM, Gardner FE (1965) The Troyer and Carrizo citranges. *Calif Citrigraph* 50:112–116
- Soost RK, Cameron JW (1975) Citrus. In: Janick J, Moore JN (eds) *Advances in fruit breeding*. Purdue University Press, West Lafayette, pp 507–540
- Vardi A, Bleichman S, Aviv D (1990) Genetic transformation of citrus protoplasts and regeneration of transgenic plants. *Plant Sci* 69:199–206
- Weigel D, Nilsson O (1995) A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377:495–500
- Wong WS, Li GG, Ning W, Xu ZF, Hsiao WLW, Zhang LY, Li N (2001) Repression of chilling-induced ACC accumulation in transgenic citrus by over-production of antisense 1-aminocyclopropane-1-carboxylate synthase RNA. *Plant Sci* 161:969–977
- Yang Z-N, Ingelbrecht IL, Louzada E, Skaria M, Mirkov TE (2000) *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). *Plant Cell Rep* 19:1203–1211
- Yang Z-N, Ye X-R, Molina J, Roose ML, Mirkov TE (2003) Sequence analysis of a 282-kilobase region surrounding the citrus tristeza virus resistance gene (*ctv*) locus in *Poncirus trifoliata* (L.) Raf. *Plant Physiol* 131:482–492
- Yao J-L, Wu J-H, Gleave AP, Morris BAM (1996) Transformation of citrus embryogenic cells using particle bombardment and production of transgenic embryos. *Plant Sci* 113:175–183
- Yu C, Huang S, Chen C, Deng Z, Ling P, Gmitter FG (2002) Factors affecting *Agrobacterium*-mediated transformation of sweet orange and citrange. *Plant Cell Tissue Organ Cult* 71:147–155



## I.3 Mango

M.A. GÓMEZ LIM<sup>1</sup> and R.E. LITZ<sup>2</sup>

### 1 Introduction

Although the mango has been in cultivation for several thousand years, today's orchards consist of vegetatively propagated selections that date only to the 16th century. According to Mukherjee (1997), the Portuguese probably introduced grafting methods into the Indian subcontinent, at which time it became possible to vegetatively propagate selections of superior seedling mango trees, including 'Alphonso', 'Dashehari', 'Langra', 'Rani Pasand' and 'Safdar Pasand'. The rapid expansion of the mango industry that has occurred worldwide in the last 25 years has largely been based upon modern selections, i.e., 'Haden', 'Keitt', 'Kent' and 'Tommy Atkins', which were identified from (uncontrolled) openly pollinated seedlings in Florida, USA. Thus, with a few exceptions, most mango cultivars have not emerged from breeding programmes. In their review of the accomplishments of conventional mango breeding, Iyer and Degani (1997) identified a number of cultivars that have been released in recent years. These include 'Amrapalli', 'Arka Anmol', 'Arka Aruna', 'Arka Puneet', 'Au-Rumani', 'Cerise', 'Heidi', 'Mallika', 'Manjiri', 'Neeleshan', 'Neeleshan Gujarat', 'Neeleshwari', 'Neelgoa', 'Neelphonso', 'Neeludin', 'Neldawn', 'Neldica', 'Ratna' and 'Swarnajehangir', amongst which only 'Mallika' has achieved consumer acceptance. For cultural reasons, there has been resistance to change from the traditional outstanding cultivars to newer selections in mango-producing countries of Southeast and South Asia.

The most significant production problems of mango cultivars include susceptibility to pre- and post-harvest anthracnose, irregular bearing and internal breakdown of fruit. As these traits have a genetic basis, a long-term solution to these problems requires a plant breeding approach. Cultivar replacement, which would involve replanting of a traditional cultivar by another cultivar, may not be satisfactory if the replacement cultivar does not have the appearance and eating quality of the traditional selection. Improvement of mangoes using conventional breeding approaches has not been a high priority in many countries, because breeding objectives cannot be achieved with this species in an expeditious manner. The mango, like most tree species, has a relatively long

<sup>1</sup> CINESTAV, Unidad Irapuato, Apartado Postal 629, Irapuato GTO 36500, Mexico, e-mail: mgomez@ciea.ira.cinvestav.mx

<sup>2</sup> Tropical Research and Education Center, University of Florida, 18905 SW 280 St., Homestead, Florida 33031-3314, USA

juvenile period of about 7 years, and the time to evaluate seedling trees can be up to 12 years. In addition, the mango is highly heterogeneous genetically, possibly tetraploid (Mukherjee 1950), and the cultivars of Southeast Asia are polyembryonic. Therefore, the efficiency of pollination is too low to permit manual, controlled pollinations, and existing collections of germplasm are often narrowly based. Although Kostermans and Bompard (1993) demonstrated that many of the wild *Mangifera* species have great potential in mango improvement programs, to date this germplasm has not been distributed widely.

Unlike conventional breeding of fruit trees, applying biotechnology to mango improvement can enable the improvement of existing cultivars by targeting specific gene traits. Improving mango by means of biotechnology is predicated upon the ability to regenerate elite selections of mango from cell and tissue cultures. Accordingly, if it is possible to genetically alter morphogenic cells derived from a mango cultivar with respect to a horticultural trait, the genetic change would also be expressed in plants that have been regenerated from those cells. Therefore, it is feasible to address production and quality problems that have a genetic basis in existing cultivars of a perennial plant. The integrity of the clone would remain unaltered except for the altered trait.

The ability to regenerate elite mango selections from cell and tissue culture also has important implications for improving plant production techniques by revolutionizing vegetative propagation. Currently, mango selections are propagated by either attached grafting (India) or unattached grafting (elsewhere) methods (Ram 1997). Air layering has also been successful in some specialized instances (Ram 1997). Propagation by cell and tissue culture techniques (micropropagation) and use of clonal rootstocks of monoembryonic types of mango could impact the study. Micropropagation could also be utilized to rapidly increase grafting scion material of compact and dwarf mango selections. Furthermore, the tissue culture protocols for regenerating mango could have a major effect on the medium- and long-term management of germplasm resources.

The other prerequisite for applying biotechnology to mango improvement is molecular biology. Molecular genetics can be applied to mango in two applications, namely, molecular markers in conventional breeding studies, i.e., marker assisted selection, and gene cloning in order to transform current mango cultivars with horticulturally important genes.

## 2 Cell and Tissue Culture

### 2.1 Shoot Tip Culture

Efforts to demonstrate a viable method of applying a standard micropropagation approach that would involve the culture of shoot tips or nodal buds of elite selections have been elusive to date with respect to mango. The advantages of this procedure include the fact that the genetic integrity of the shoot

apex would be preserved and juvenility would not be reintroduced into the clone, causing delayed flowering in the field. An important limitation of the application of shoot tip and nodal culture to mature phase mango appears to be the rapid oxidation of mango tissues following their removal from the plant. Consequently, the only report of the micropropagation of mango has involved juvenile phase material, i.e., 2-year-old seedlings, of four polyembryonic cultivars, 'Gomera', 'Sabre', 'Terpentine' (sic.) and '13-1' (Yang and Ludders 1993). In this report, a complex plant growth medium, consisting of G formulation, supplemented with 4.4  $\mu\text{M}$  benzyladenine (BA), 4.6  $\mu\text{M}$  zeatin, 9.8  $\mu\text{M}$  (2-Isopentenyl) adenine, 2.9  $\mu\text{M}$  indole-3-acetic acid (IAA), 2.5  $\mu\text{M}$  indole-3-butyric acid (IBA), 300  $\text{mg l}^{-1}$  glutamine and 300  $\text{mg l}^{-1}$  casein hydrolysate was utilized to stimulate proliferation of axillary buds from explanted shoot tips. On this formulation, 'Terpentine' (sic.) produced the greatest number of shoots. There was a pronounced seasonal effect on the establishment of explants, with the highest frequency of establishment obtained during June and July under northern European conditions. Rooting of microcuttings was not attempted. Although this procedure involved the use of seedling shoot tips, their origin from polyembryonic mango seedlings indicates that they were most probably clonal in origin.

## 2.2 Organogenesis

The first report of organogenesis in mango tissue cultures involved a study in which cotyledons were removed from mango embryos, and were explanted onto MS medium (Murashige and Skoog 1962) supplemented with 15% (v/v) coconut water, 26.9  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid (NAA) and 11.6–23.2  $\mu\text{M}$  kinetin (Rao et al. 1982). On this medium, a dark globular callus mass formed, from which adventitious roots developed. There was no shoot development.

Relatively young 3- to 5-cm explants from mature phase trees have also been used for obtaining organogenic cultures of mango (Raghuvanshi and Srivastava 1995). 'Amrapali' leaves were explanted under axenic conditions into sterile liquid MS medium supplemented with 0.05% polyvinylpyrrolidone in order to control oxidation of the tissue. After shaking for up to 24 h, the leaf tissue was cultured on MS medium supplemented with different plant growth regulators (PGRs), namely 2.2–17.8  $\mu\text{M}$  BA with 0.6–5.0  $\mu\text{M}$  NAA, or 1.1–5.7  $\mu\text{M}$  IAA and 3.3–26  $\mu\text{M}$  kinetin with 0.6–5.0  $\mu\text{M}$  NAA or 1.1–5.7  $\mu\text{M}$  IAA. On most PGR combinations, callus with organogenic competence developed from the leaf explants and adventitious shoots developed from the callus. The individual shoots were rooted in vitro on medium containing 9.8  $\mu\text{M}$  IBA.

## 2.3 Somatic Embryogenesis

De novo regeneration can also involve the formation of somatic embryos from certain types of tissues that are explanted at specific developmental stages.

Embryogenic competence is normally mediated by several factors in addition to the type and stage of development of the explant. For example, the response is strongly genotype-dependent, and has been shown to be inherited in a Mendelian manner in *Dactylis glomerata* (Gavin et al. 1989). The composition of the induction medium is often critical. Key variables include the basal medium composition, the presence of organic or inorganic reduced nitrogen, osmolarity and a strong artificial auxin, i.e., 2,4-D, picloram or dicamba.

Mango is perhaps unique amongst plant species, in that it consists of two ecogeographic races that can be distinguished on the basis of their seed type, i.e., monoembryonic/subtropical and polyembryonic/tropical. The presence of adventitious nucellar embryos in polyembryonic mango seeds is an indicator that the nucellus has embryogenic potential. Maheshwari and Rangaswamy (1958) attempted to induce embryogenic competence in mango ovules in vitro, but were not successful. Litz et al. (1982) first described the production of somatic embryos after explanting polyembryonic mango ovules onto MS medium containing various PHRs. In later reports, the initiation of embryogenic suspension cultures was described (Litz et al. 1984) and embryogenic cultures were induced from monoembryonic mango cultivars (Litz 1984). DeWald et al. (1989a) characterized the initiation and maintenance of embryogenic cultures, and described the basal medium formulation that is now used routinely for induction and maintenance of embryogenic mango cultures. This medium includes B5 (Gamborg et al. 1968) major salts [without  $\text{NH}_4(\text{SO}_4)_2$ ], MS minor salts and organic components, 400 mg l<sup>-1</sup> glutamine, 60 g l<sup>-1</sup> sucrose, 4.5 µM 2,4-D and 2.0 g l<sup>-1</sup> gellan gum. For induction, the medium is semi-solid, while liquid formulation is utilized for medium-term maintenance. Later, DeWald et al. (1989b) described the parameters for initiating somatic embryo development (maturation) and germination. These plant growth medium formulations are still being utilized with minor modifications (Litz et al. 1993, 1995; Litz and Lavi 1997).

The standard protocol for establishing embryogenic cultures involves the use of young mango fruit 30–40 days after flowering. It is important to identify the stage of development of the immature seed, because the nucellar tissue is highly vulnerable to wounding early in development, and can be rapidly consumed by the developing embryo(s). In general, the embryo mass should be half to almost the same length as the embryo sac. In polyembryonic seeds the embryo mass generally develops more rapidly than that in monoembryonic seeds. The entire fruit is surface-disinfected and washed with sterile deionized or double-distilled water to initiate embryogenic culture. Under axenic conditions, the fruit is bisected along its longitudinal axis without damaging the immature seed, which is carefully removed. The immature seed is also bisected along its longitudinal axis, and the embryo mass is removed and discarded. The nucellus, particularly at the micropylar end, is a thick tissue, and can be removed with a sterile flat spatula and transferred onto semi-solid induction medium (see earlier) in a Petri dish. Alternatively, the immature seed halves with attached nucellus can be placed in culture to allow direct contact of the nucellus with

the induction medium. The cultures are incubated in darkness at 25 °C, and subcultured daily until the medium is no longer discolored around the explant.

According to Lad et al. (1997), the temporal requirement for acquisition of embryogenic competence from mango nucellar cultures is about 28 days with 'Carabao', and a low frequency of somatic embryogenesis can be observed from the nucellus of both monoembryonic and polyembryonic cultivars in the absence of PGRs (Lad et al. 1997; Litz et al. 1998). Initially embryogenic cultures are friable and callus-like. These cultures are comprised entirely of proembryonic cells or masses that are committed to somatic embryogenesis.

Genotype strongly affects the embryogenic response and subsequent maintenance of embryogenic cultures. As a consequence, it is difficult to generalize the response of explanted nucellar cultures. Litz et al. (1998) demonstrated that the embryogenic potential was genotype-dependent by culturing the nucellus of four mango cultivars on induction medium. The embryogenic potential of the cultures in declining order were 'Hindi' (polyembryonic), 'Lippens' (monoembryonic), 'Tommy Atkins' (monoembryonic) and 'Nam doc Mai' (polyembryonic). Embryogenic competence was not related to either monoembryony or polyembryony. Induction could also be enhanced in the three cultivars 'Hindi', 'Lippens' and 'Nam doc Mai' if a nurse culture consisting of embryogenic 'Parris' was utilized. Rivera Domínguez et al. (2004) recently reported official induction of somatic embryogenesis and plant regeneration in the cultivar 'Ataulfo'. It is possible that the sensitivity of nucellar explants to ethylene is an important key to induction. Litz and Yurgalevitch (1997) demonstrated that induction of competence of nucellar explants of monoembryonic mango in comparison with polyembryonic mango could be suppressed by the ethylene antagonists AVG (aminoethoxyvinylglycine) and DCHA (dicyclohexylammonium sulfate). However, induction of embryogenic competence was also suppressed in mono- and polyembryonic mangoes by ACC (1-aminocyclopropane-1-carboxylic acid), a precursor of ethylene biosynthesis. Litz and Yurgalevitch concluded that induction of embryogenic competence was more sensitive to spermidine synthase suppression than to S-adenosylmethionine (SAM) decarboxylase activity. Litz and Schaffer (1987) observed that spermidine concentrations were higher in the nucellus of polyembryonic than in monoembryonic genotypes, and that exogenously applied spermidine is more effective in inducing embryogenic cultures from cultured nucellus of polyembryonic than monoembryonic mango cultivars.

Embryogenic cultures that are maintained on induction medium are inhibited by the presence of 2,4-D, and cannot develop as mature somatic embryos. Consequently, globular somatic embryos enlarge, lose their integrative ability and become proembryonic masses. Proembryonic masses continue to proliferate under inductive conditions by the formation of secondary globular embryos from single cells in the protoderm region of older proembryonic masses (Litz et al. 1993, 1995; Litz and Lavi 1997).

Embryogenic cultures of most mango genotypes can be maintained more efficiently in suspension culture than on semi-solid medium. Embryogenic

suspension cultures can be established by inoculating 300–500 mg of embryogenic culture into 40-ml sterile liquid medium in 125-ml Erlenmeyer flasks maintained at 120 rpm and 25 °C in semi-darkness. It is essential that these cultures are transferred to new medium at weekly intervals. Cultures cannot be maintained indefinitely in maintenance medium, due to their gradual loss of embryogenic competence. However, 'Hindi' has been maintained for more than 4 years without losing its regenerative potential. It has been observed that some mango cultivars, e.g., 'Keitt', cannot be maintained in liquid medium (Litz et al. 1993).

It is necessary to subculture from liquid medium with 2,4-D to the same medium without 2,4-D to promote somatic embryo development or maturation. For somatic embryo maturation, a sequence of medium formulations has been utilized. Initially, the basal medium (described earlier) containing 4.7 13.9  $\mu\text{M}$  kinetin is important for stimulating the organization of cotyledon primordia. The heart stage somatic embryos are usually present from 30–35 days ('Hindi') to 60–70 days after subculture ('Lippens', 'Tommy Atkins', 'Sabre') (Litz et al. 1993, 1995). Somatic embryos at early cotyledonary stage are subcultured onto semi-solid basal medium containing 20% (v/v) filter-sterilized coconut water and 30  $\text{g l}^{-1}$  sucrose in the absence of PGRs. DeWald et al. (1989b) demonstrated that 3.8–11.4  $\mu\text{M}$  ABA was beneficial for maturation of mango somatic embryos, which were able to develop to maturity and germinate in the dark.

Suspension cultures of some mango cultivars, e.g., 'Hindi', proliferate very rapidly, and develop extensively and quickly as cotyledonary embryos following transfer to 2,4-D-free medium. The heart stage somatic embryos of these cultivars are often hyperhydric, and are incapable of developing to maturity. Monsalud et al. (1995) developed two procedures to reverse hyperhydricity of mango somatic embryos. These procedures included exposure of hyperhydric somatic embryos to low relative humidity, and pulsing the hyperhydric somatic embryos with >750  $\mu\text{M}$  ABA. When they are fully mature, somatic embryos are 4–6 cm in length.

Germination is signaled by the elongation of the hypocotyl and growth of the tap root. The cultures are transferred to light conditions with a 16-h photoperiod provided by cool white fluorescent lights (30  $\text{mol m}^{-2}\text{s}^{-1}$ ). The shoot emerges about 2 weeks later. In order to recover regenerants that are photoautotrophic, young mango plantlets have been transferred to medium in G7 containers, the latter being covered with lids modified to hold a sterile Suncap (Sigma Co., St. Louis, Missouri) gas permeable filter to permit gas exchange. The medium consists of basal medium with 0.3% (w/v) activated charcoal and 20  $\text{g l}^{-1}$  sucrose. The G7 containers are placed in the chamber with 20,000 ppm  $\text{CO}_2$  and an  $\text{N}_2$  carrier is passed through, with a light intensity of 130–140  $\mu\text{mol m}^{-2}\text{s}^{-1}$  provided by cool white fluorescent lamps.

Somatic embryos have been successfully recovered from several mango cultivars (Table 1).

**Table 1.** Induction of somatic embryogenesis from nucellar cultures of mango (Jana et al. 1994; Litz and Lavi 1997)

Cultivar	Seed type	Cultivar	Seed type	Cultivar	Seed type
Alphonso	mono	Heart	poly	Mulgoa	mono
Arumanis	poly	Hindi	poly	Mundan	mono
Baneshan	mono	Honc Cambodiana	poly	Nam doc Mai	poly
Brander	poly	Irwin	mono	Neelum	mono
Brooks	mono	James Saigon	poly	Ono	poly
Cambodiana	poly	Keitt	mono	Parris	poly
Carabao	poly	Kensington	poly	Peach	poly
Chino	poly	Kur	poly	Philippine	poly
Everbearing	mono	Langra Benarsi	mono	Simmonds	poly
Dashehari	mono	Lippens	mono	Sabre	poly
Florigon	poly	Madu	poly	Tommy tkins	mono
Gedong	poly	Manzano	poly	Tuehau	poly
Golek	poly	Mikongogenesis	poly	Turpentine	poly
				White Langra	mono

## 2.4 Potential for Micropropagation and Conservation

The potential of in vitro protocols for micropropagating mango is limited by the relative ease with which mango can be propagated using conventional vegetative methods. Therefore, it is likely that these techniques will only be used for highly specialized purposes, such as to rapidly propagate transgenic plants, propagation of clonal rootstocks of monoembryonic types of mango, propagation of dwarf and compact mango selections and conservation of mango genetic resources.

## 2.5 Germplasm Conservation

There are relatively few comprehensive germplasm collections of mango and related species. In an ex situ germplasm collection, each accession must be replicated and, ideally, the collection should be duplicated in case of a natural disaster. Because of the large number of mango cultivars and *Mangifera* species (Kostermans and Bompard 1993), the cost of establishing and maintaining such collections can be prohibitively high. This limits the gene pool available to plant breeders, plant pathologists and other researchers of this crop.

Alternatives to ex situ collections include the use of slow-growing in vitro cultures for short- and medium-term storage and the use of seed banks and cryopreservation of in vitro cultures for long-term storage. Mango seeds are recalcitrant, and are therefore unable to withstand maturation desiccation. In view of this, mango seeds cannot be stored in a traditional seed bank because



of their rapid loss in viability. Parrisot (1988) demonstrated that mango seeds could be stored for only a few days under room conditions and for a few weeks at temperatures that were suboptimal for growth and development. When storing seeds of fruit trees, it is also important to remember that their seeds are not clonal, with the exception of those cultivars having polyembryonic seeds.

In order to investigate whether mango somatic embryos could be utilized for storage of clonal mango germplasm, Pliego-Alfaro et al. (1995a, b) examined various parameters, including temperature, ABA concentration and osmolarity, that limit growth and development of embryos. The treatments were undertaken in order to cause developmental arrest in mango somatic embryos, and to attempt to induce desiccation tolerance. It was found that the development of late heart stage somatic and nucellar embryos was suppressed at 7.5 °C, but development was resumed after cultures or transfer to 25 °C. Embryo development was also arrested by 4- and 8-week pulses of 1,000 µM ABA, which exerted a significant residual effect for up to 3 months. Treatment with 7.5% mannitol also caused developmental arrest of mango embryos, but there was no residual effect. When mannitol was applied in conjunction with ABA, there was no interaction of the two treatments, and embryo development was only inhibited in the presence of high ABA concentrations. Pulsing with ABA and mannitol did not confer desiccation tolerance. However, untreated late heart stage somatic embryos could survive partial desiccation after the loss of 70% fresh weight, and remained viable for up to 32 days when stored dry under sterile conditions (in the absence of growth medium) (Monsalud et al. 1995). These results indicate that desiccated heart stage embryos are stored like orthodox seeds under appropriate conditions. According to Engelmann (1991) and Withers (1992), embryogenic cultures of recalcitrant seed species can be cryopreserved without loss of viability. Wu et al. (2003) compared three cryopreservation protocols for embryogenic 'Zihua', including encapsulation-dehydration, pregrowth- dehydration and vitrification. The first protocol was unsuccessful and only 8.3% of embryos survived following desiccation for 1 h to 58% moisture content prior to freezing in liquid nitrogen. Nevertheless, this strategy has great potential for future studies.

### 3 Applications of Biotechnology

#### 3.1 In Vitro Selection

Although embryogenic suspensions comprise cells and proembryonic masses that are theoretically genetically uniform, it has been shown that genetic mutations can occur during in vitro cycles, i.e., somaclonal variation (Larkin and Scowcroft 1981). Since the variation is random and nondirected, the screening of regenerated plants in the field for useful somaclonal variants is tedious and inefficient (Hammerschlag 1992). Under certain circumstances, it is possible



to screen and select for a specific trait at the cell level. In many pathogen–host interactions, the pathogen produces a phytotoxin that can induce symptoms at the single and whole plant level, e.g., bacterial leaf spot of peach *Prunus persica* caused by *Xanthomonas campestris* pv. *pruni* (E.F. Sm.) (Hammerschlag 1992).

Anthrachnose in mango is caused by *Colletotrichum gloeosporioides* Penz., and is the major problem in mango production and post-harvest in many regions. *Colletotrichum gloeosporioides* has been demonstrated to produce at least three phytotoxins, i.e., colletotrichin (Gohbara et al. 1978), lycomaras-mic acid and aspergillomarasmine (Ballio et al. 1969; Bosquet et al. 1971) during infection of susceptible plant tissues. Jayasankar et al. (1998) reported that embryogenic cultures of elite mango cultivars, i.e., ‘Carabao’ and ‘Hindi’, could be selected for resistance to culture filtrate, and the selected lines could strongly suppress growth of *C. gloeosporioides* in vitro (Jayasankar and Litz 1998). The selected ‘Carabao’ and ‘Hindi’ lines produced newly expressed extra-cellular pathogenesis-related (PR) proteins, e.g., chitinase and  $\beta$ -1,3-glucanase isozymes. The extracellular secretion of chitinase and  $\beta$ -1,3-glucanase by plant tissues has been associated with pathogen attack (Lamb et al. 1992), and may act as an early line of defense in plants by blocking growth of fungal hyphae (Collinge et al. 1993). The DNA of selected lines, controls and parent trees was analyzed using RAPD, and markers were identified with eight primers that were either present or absent in the selected lines relative to the controls and the parent trees (Jayasankar et al. 1998). This strongly indicated that mutations in vitro were induced during in vitro selection, since the controls were genetically stable relative to the parent trees. Overproduction of chitinase and  $\beta$ -1,3-glucanase was genetically stable, and strong expression was detected in culture medium more than 1 year after cessation of selection pressure.

### 3.2 Genetic Transformation

Production of pathogen-resistant plants can also be achieved by transferring the genes coding for antifungal proteins or PR proteins into the plants, as reported by Gómez Lim (2001) and Campbell et al. (2002).

The transformation of mangoes was reported by Mathews and Litz (1990) and Mathews et al. (1992, 1993). Embryogenic cultures derived from cultured zygotic embryos of ‘Keitt’ were transformed with disarmed *Agrobacterium tumefaciens* strain C58CI containing the vector pGV3850::1103 with the *nptII* gene. Transgenic plants were recovered from transformed somatic embryos (Mathews et al. 1993). Embryogenic cultures derived from the nucellus of ‘Hindi’ were transformed with the disarmed *A. tumefaciens* strain A208 containing pTiT37-SE::pMON (9749 ASE) with the *nptII* and *gus* genes. Although transformed somatic embryos were obtained, plantlets were not recovered (Mathews et al. 1992). In these studies, stable expression of the genes was demonstrated, and insertion of the genes into the host genome was confirmed by Southern analysis.

### 3.3 Metabolic Manipulation by Genetic Transformation

#### 3.3.1 Fruit Ripening

One of the major constraints on increasing the yield of many fruit crops has been the premature, ethylene-induced ripening of the fruits. Most fruits are highly perishable and have a limited shelf life. This may cause several problems with respect to transportation and marketing, as about 50% of all fresh fruits and vegetables may be lost due to such spoilage. Post-harvest problems have been partially solved for many commercial crops, particularly those grown in temperate climates, by harvesting them at the immature or green stage, and/or by storage at low temperatures or in controlled atmospheres. However, these practices are not effective with regard to tropical fruits including mangoes. Mangoes harvested at full maturity do not store well, and fail to ripen if harvested when immature. In addition, mangoes are very susceptible to low temperature.

A major goal in the genetic engineering of fruit quality is the control of ethylene production and action because ethylene plays a key regulatory role in fruit ripening (Carrari and Fernie 2006). Two different strategies have been employed to achieve this aim. One involves inhibition of expression of the genes encoding ethylene biosynthetic enzymes by transformation with the respective antisense genes, while the other strategy is to lower the level of endogenous ACC by deamination or hydrolysis. Ethylene biosynthesis is catalyzed by three major enzymes, SAM synthetase, ACC synthase and ACC oxidase. The last two are the key enzymes in the pathway and these genes have been cloned from a number of plant species (Zarembinski and Theologis 1994). Transformation with antisense ACC synthase and ACC oxidase has resulted in delayed ripening of tomato fruits, permitting longer vine ripening while retaining good shipping qualities (Oeller et al. 1991; Gray et al. 1992).

Two cDNA clones have been identified as ACC synthase and ACC oxidase from a mango cDNA library (Gómez Lim 1997a). ACC synthase expression was not detectable in unripe fruit, but the transcript began to accumulate in ripening fruit and the concentration reached a maximum in ripe fruit (Gómez Lim 1997a). This pattern of expression is similar in both the peel and pulp during ripening, but transcript in the pulp was detected earlier than in the peel. ACC oxidase showed a similar kinetics in both tissues, and the transcript was clearly detectable before ACC synthase expression (Gómez Lim 1997b). These results suggest that ACC oxidase is expressed before ACC synthase and that ripening starts in the pulp and proceeds outward in mango fruit, which is consistent with the results shown in other fruits such as banana (Domínguez and Vendrell 1994). Interestingly, ethylene-treated fruits show a different pattern of expression, with ACC oxidase and ACC synthase first appearing in the peel. In melon, fruit ripening was delayed by expression of the antisense of ACC oxidase RNA (Ayub et al. 1996). Although this methodology has general applicability, it has not been extended to many other crop species.

### 3.3.2 Lipid Metabolism

Genetic engineering of the lipid composition in higher plants can lead to improvement in the nutritional value of crops and enhancement of plant capability to withstand low temperature stress. The identification of desaturases, enzymes that introduce a *cis*-double bond in saturated fatty acids, has led to the production of plants with increased concentrations of polyunsaturated fatty acids (Arondel et al. 1992) or increased chilling tolerance (Ishizaki-Nishizawa et al. 1996). Although mango fruits do not store lipids, these compounds have been associated with color and flavor development during ripening. The vast majority of lipids found in mango are esters of long chain fatty acids. In mango fruits, the fatty acid oxidizing activity increases with ripening (Baqui et al. 1977), while several fatty acids (particularly linoleic and oleic acids) decrease during ripening. Products of  $\beta$ -oxidation are utilized in the synthesis of both carotenoids and terpenoid volatiles, which are the important aroma components of mango fruits. Interestingly, Bojórquez and Gómez Lim (1995) reported that mRNA of peroxisomal thiolase, an enzyme of the  $\beta$ -oxidation pathway, is induced during fruit ripening of mango. These results indicate that increased activity in the  $\beta$ -oxidation pathway enzymes during ripening is important for aroma production. Recently, expression of a mango Acyl-CoA oxidase, a key enzyme in  $\beta$ -oxidation, isolated in our laboratory, was found to induce fruit ripening (unpublished results). These results indicate the possible role of these enzymes in metabolizing fatty acids to produce volatile compounds.

### 3.3.3 Plant Architecture

Modification of plant architecture by genetic means is now a reality. The phytochromes are a family of photoreceptors that function as photoreversible pigments in plants. Robson et al. (1996) reported the production of transgenic plants over-expressing phytochrome A. The transgenic plants were tested in the field and they displayed suppression of the shade-avoidance mechanism. Under normal conditions, plants respond to the enhanced level of far red light in the shade of other plants by excessive elongation. In commercial glasshouses, this leads to rapid elongation of potted plants, which frequently requires application of chemical growth retardants. These transgenic plants appeared not to require chemical retardants. In addition, some of the transgenic lines showed a reduced stature in the field. In the case of mango, plants with reduced stature would facilitate harvesting. Leaves of transgenic plants with up to 30% more chlorophyll may result in increased photosynthesis. The transgenic plants also accumulated significantly more biomass (Robson and Smith 1997). A clone encoding a mango phytochrome gene has recently been identified from a mango genomic library and it is presently being characterized (Gómez Lim 1998).

### 3.3.4 Flower Formation

Flower development is an unpredictable and irregular process in many commercial crops. Applications of several chemicals are required to stimulate and coordinate mango flower formation. Molecular genetic studies have shown that at least three classes of homeotic genes control the determination of floral meristems and organ identity in higher plants (Weigel and Meyerowitz 1994). Transgenic plants overexpressing the *LEAFY* (Weigel and Nilsson 1995) and *CONSTANS* (Simon et al. 1996) genes have been produced. These genes are sufficient to determine floral fate in lateral shoot meristems, with the consequence that flower development is induced precociously. This phenotype was also reported in plants over-expressing the phytochrome genes (Robson and Smith 1997). In future, cloning and engineering homologous genes in mango may lead to improved control of the flowering process.

### 3.3.5 Reduction of the Juvenility Period

Mango, like most tree species, has a long juvenile period of about 7 years, and the time to evaluate seedling trees can be up to 12 years. This has hampered the development of new, improved cultivars by traditional plant breeding and poses a challenge for the generation of improved varieties when using plant tissue culture techniques. Transgenic citrus plants over-expressing the genes *LEAFY* or *APETALA1*, which promote flower initiation in *Arabidopsis*, have been produced (Peña et al. 2001). Both types of transgenic citrus produced fertile flowers and fruits as early as the first year, and a shortening of their juvenile period was observed. Furthermore, expression of *APETALA1* was as efficient as *LEAFY* in flower initiation, and did not show severe developmental abnormality. Both types of transgenic trees flowered in consecutive years, and their flowering response was under environmental control. In addition, zygotic- and nucellar-derived transgenic seedlings had a very short juvenile phase and flowered in their first spring, demonstrating the stability and inheritance of this trait. These results have opened up new avenues for research in the genetic improvement of mango.

### 3.3.6 Fruit Quality

Fruit quality refers to color, texture and flavor. The external color of fruit is an important factor in consumer preference. The principal pigments in mango fruit are chlorophylls, carotenes, xanthophylls and anthocyanins, which are synthesized via the terpenoid or phenylpropanoid pathways. To date, there is only one example of genetic manipulation of fruit color by the change in phytoene synthase. Phytoene synthase catalyzes the dimerization of two molecules of geranylgeranyl pyrophosphate to form phytoene, the first C40 carotene in the carotenoid synthesis pathway. Expression of antisense phytoene synthase

RNA in tomatoes produced pale yellow flowers and fruits that ripened to a yellow color (Bird et al. 1991). Lycopene could not be detected in those fruits, although other aspects of ripening, such as polygalacturonase accumulation, were unaffected. Manipulation of pigment synthesis by genetic engineering represents an attractive strategy to modify the fruit color of mango in future research.

Ripening of mango is characterized by softening of the flesh. Softening is caused by a series of changes in the metabolism and structure of cell walls, which results in a change in texture. The ripening of fruits is accompanied by fragmentation of pectic polymers and hemicelluloses, solubilization of long-chain pectin and the loss of specific sugars, e.g., galactose and arabinose, in the walls (Huber 1983). These modifications reduce wall strength and cell-to-cell adhesion, leading to softening and the characteristic texture of many ripened fruits.

Attempts have been made to investigate the role of polygalacturonase (PG) in fruit softening by expression of antisense PG mRNA in tomatoes (Sheehy et al. 1988; Smith et al. 1988). In transgenic plants, the levels of PG mRNA, protein and enzyme activity were reduced in fruits to approximately 10% of that in wild-type fruit. However, phenotypic analysis of transformed and non-transformed fruits revealed no obvious differences in compressibility, ethylene production or lycopene accumulation (Sheehy et al. 1988; Smith et al. 1988), suggesting that PG-mediated depolymerization of pectins is not essential for softening and for other fundamental processes associated with fruit ripening. Similar results have also been reported by Giovannoni et al. (1989). This is in contrast to considerable correlative data linking PG-mediated pectin hydrolysis to ripening-related softening (Giovannoni 2001). Using a similar approach, the levels of pectinmethylesterase (PME) mRNA, protein and enzyme activity were greatly reduced in transgenic fruit expressing antisense PME gene (Tieman et al. 1992; Hall et al. 1993). Although there were no major differences in fruit development and ripening between transformed and wild-type fruits, pectin remained more heavily esterified (methylated) at all stages of development. Tieman et al. (1992) also generated transgenic tomato over-expressing the PME gene. Although transformed fruits showed highly variable PME activity that in some cases reached up to 140% of that in wild-type fruit, softening was unaffected. Similarly, fruit softening was not affected in transgenic plants expressing the chimeric PG/PME gene in which a 244-bp of the 5' end of PG cDNA fused to a 1320-bp of PME cDNA (Seymour et al. 1993). Pear et al. (1993) also transformed tomatoes with antisense PG and PME genes, but softening was not altered significantly, although there was a significant reduction in PG and PME mRNA and enzyme activity. These results suggest that softening may be due primarily to the concerted action of several cell wall hydrolases in addition to PG and PME, whose activities change during ripening. It is speculated that degradation of starch and loss of turgor may also contribute to softening, although the exact mechanism is not clear.

Apart from PG PME and the other cell wall hydrolases,  $\beta$ -galactosidase may play a role during fruit ripening. A cDNA coding for  $\beta$ -galactosidase isolated from mango (Gómez Lim 1998) showed an increase in mRNA, which was correlated with the increase in  $\beta$ -galactosidase activity as ripening proceeded. Southern analysis suggested that the  $\beta$ -galactosidase gene may be present in more than one copy in the mango genome, which may explain the presence of several  $\beta$ -galactosidase isozymes in mango (Ali et al. 1995).

A partial cDNA clone coding for a mango xyloglucan endo-transglycosylase has been identified (GenBank accession no. AY600965). Xyloglucan endo-transglycosylase is a cell wall hydrolase, which catalyzes the cleavage and concomitant transfer of one xyloglucan molecule to another. The enzyme is thought to be involved in cell wall metabolism, particularly in expanding tissue and ripening fruit. In tomato, there was an increase in xyloglucan endo-transglycosylase activity during fruit ripening (Arrowsmith and de Silva 1995). Chanliaud et al. (2004) demonstrated that the mechanical consequences of the action of this enzyme are complementary to those of expansin.

Expansin comprises a large family of enzymes, the expansions, involved in cell wall modification (Cosgrove et al. 2002). These enzymes have also been found in ripening fruits, and over-expression or antisense inhibition in tomato fruit resulted in a drastic change in texture and processing characteristics (Kalamaki et al. 2003; Powell et al. 2003). A partial cDNA clone coding for a mango expansin has been identified (GenBank accession no. AY600964), which suggests a possible role for these enzymes in mango fruit softening.

No major advances have been made in the identification of flavor components and their enzymes in tropical fruits. This might be a reflection of the complexity of this trait. Since mangoes contain large quantities of carbohydrates, particularly sucrose, genetic manipulation of the sucrose-metabolizing enzymes may provide a way to alter sugar content and, in turn, fruit flavor. There are several reports on the manipulation of different enzymes involved in carbohydrate metabolism. Transgenic plants have been produced that over-expressed and/or down-regulated the expression of genes encoding carbohydrate metabolism enzymes. These enzymes include invertase (Zrenner et al. 1996; Sonnewald et al. 1997), granule-bound starch synthase (Kuipers et al. 1994), phosphorylase (Duwenig et al. 1997), ADP-glucose pyrophosphorylase (Rober et al. 1996), sucrose synthase (Zrenner et al. 1995), uridine diphosphate-glucose pyrophosphorylase (Zrenner et al. 1993) and sense sucrose phosphate synthase (Worrell et al. 1991). Apart from modification of carbohydrate metabolism, another approach to enhance fruit flavor is the expression of sweet-tasting proteins, such as monellin or thaumatin. These proteins elicit a sweet flavor by binding specifically with taste receptors and are approximately 100,000 times sweeter than sugar on a molar basis (Van der Wel and Arvidson 1978). Transgenic tomato fruits have been reported that over-expressed monellin (Peñarrubia et al. 1992). Thaumatin has been shown to be strongly induced in ripening fruits (Fils-Lycaon et al. 1996; Tattersall et al. 1997). All transgenic plants showed an altered carbohydrate content.



### 3.3.7 Disease Resistance

Genetic transformation is an attractive alternative to producing pathogen-resistant transgenic plants. There are different types of antifungal proteins, some of which have been utilized to confer resistance against different plant pathogens. The availability of genes coding for pathogenesis-related (PR) proteins, i.e., chitinase and  $\beta$ -1,3-glucanase, has allowed the generation of plants that constitutively express different PR proteins and show resistance to fungal infection (Gómez Lim 2001). However, greater protection can be achieved when more than one PR protein is employed (Gómez Lim 2001). Other antifungal proteins include ribosome inactivating proteins (RIP) that inhibit protein synthesis by specific RNA N-glycosidase modification of 28S rRNA. RIPs inactivate ribosomes of distantly related species, including fungi. Transgenic plants expressing RIPs are resistant to fungal pathogens (Logemann et al. 1992), while over-expressing chitinase, glucanase and RIPs also leads to increased fungal protection (Jach et al. 1995) compared with the protection obtained with corresponding transgenic lines expressing a single transgene to a similar level. These results indicate synergistic protective interaction of the co-expressed antifungal proteins *in vivo*. In addition to RIPs, one group of cysteine-rich, small molecular weight (5-kDa) proteins, namely defensins, has been shown to be an important element in plant defense against fungal attack (Broekaert et al. 1995). Work is presently progressing in several laboratories around the world to generate transgenic plants expressing the defensin genes.

### 3.4 Other Characteristics

Apart from manipulation of the metabolic processes by down-regulation and over-expression of genes for the key enzymes in the pathway, other genes identified from mango can also be used for its improvement. These genes include alternate oxidase, ethylene receptor and a rab11-like gene. Alternate oxidase is an enzyme involved in the cyanide-resistant respiratory pathway. It has been studied mainly in thermogenic species and its activity is correlated with heat production, which is used to volatilize foul-smelling compounds to attract insect pollinators. In mango, alternate oxidase gene expression could be detected in both unripe and ripe fruits, but expression was substantially greater in the latter (Cruz Hernandez and Gómez Lim 1995). The level of transcript accumulation was correlated with that of enzyme activity and protein. It has been reported that alternate oxidase may be responsible for higher temperature in ripe mango pulp, up to 10 °C, compared to unripe pulp (Kumar et al. 1990). This extra heat may also serve to volatilize aromatic compounds. A mango cDNA has been reported for an ethylene receptor (Gutiérrez Martínez et al. 2001). This gene has low expression in unripe fruit, but the transcript level increased markedly in ripe fruit and in response to wounding. However, the role of ethylene receptor in mango ripening remains to be determined.

Zainal et al. (1996) reported a mango rab11/YPT3 cDNA whose expression was ripening-inducible. This gene belongs to a family of small GTPases that is thought to be involved in the control of protein trafficking within cells. In fruits, they may have a possible role in trafficking of cell wall-modifying enzymes. In tomato, transgenic fruit expressing antisense rab11-like cDNA showed color change, but failed to soften normally (Lu et al. 2001). This was accompanied by reduced concentrations of pectin esterase and PG. Transgenic plants also displayed other phenotypic changes, including determinate growth, reduced apical dominance, branched inflorescences, abnormal floral structure, and ectopic shoots on the leaves. Ethylene production was reduced in some plants. These results suggest the possible role of rab11-like gene in fruit ripening.

In view of the large number of mango microsatellite sequences available in the GenBank, parameters such as heterozygosity, average gene diversity, frequency of outcrossing, cultivar relationships and a mango genetic map will be determined in the near future. Correlations between morphological and DNA markers, together with a linkage map, should eventually enable marker-assisted selection for mango improvement. Furthermore, genomic tools are being employed in mango and over 1000 ESTs have been sequenced recently. These ESTs are also being analyzed by microarrays using RNA probes from different ripening stages and from different mango cultivars (unpublished results). The availability of tissue-specific promoters, e.g., fruit-specific and cell wall-specific, is important for mango improvement using genetic engineering. For this reason, the genomic clones for  $\beta$ -galactosidase (fruit-specific) and a photosynthetic protein (leaf-specific) are currently being characterized (unpublished results).

It is noteworthy that there have been several recent reports on the therapeutic properties of mango extracts that possess anti-inflammatory (Beltrán et al. 2004; Garrido et al. 2004; Leiro et al. 2004), anthelmintic and antiallergic (García et al. 2003a), and antidiarrhoeal (Sairam et al. 2003) characteristics, and an enhancement of the humoral immune response in mouse (Garcia et al. 2003b). It will be possible to enhance these therapeutic effects in the future if the genetic elements for the active ingredients in the extract are identified.

## 4 Conclusions

Biotechnology tools have great potential for mango, including advanced micropropagation procedures, conservation and cultivar improvement. Most of this research has been carried out in a few research centers. However, it is now widely acknowledged that biotechnology is mainstream research. It is time for other countries to join this research field.

**Acknowledgements.** The authors wish to thank the Florida Agricultural Experiment Station Journal Series no. R-10752. M.A.G.L. also acknowledges the continuous support of CONACYT.



## References

- Ali ZM, Armugam S, Lazan H (1995)  $\beta$ -Galactosidase and its significance in ripening mango fruit. *Phytochem* 38:1109–1114
- Aronel V, Lemieux B, Hwang I, Gibson S, Goodman HM, Somerville CR (1992) Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science* 258:1353–1355
- Arrowsmith DA, de Silva J (1995) Characterisation of two tomato fruit-expressed cDNAs encoding xyloglucan endo-transglycosylase. *Plant Mol Biol* 28:391–403
- Ayub R, Guis M, Ben-Amor M, Gillot L, Roustan JP, Latché A, Bouzayen M, Pech JC (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. *Nat Biotechnol* 14:862–865
- Ballio A, Bottalico A, Buonocore V, Carilli A, Di Vittorio V, Graniti A (1969) Production and isolation of aspergillomarasmine B (lycomarasmic acid) from cultures of *Colletotrichum gloeosporioides* Penz. (*Gloeosporium olivarum* Alm.). *Phytopath Medit* 8:187–196
- Baqui SM, Mattoo AK, Modi VV (1977) Glyoxylate metabolism and fatty acid oxidation in mango fruit during development and ripening. *Phytochemistry* 13:2049–2055
- Beltrán AE, Alvarez Y, Xavier FE, Hernanz R, Rodriguez J, Nunez AJ, Alonso MJ, Salas M (2004) Vascular effects of the *Mangifera indica* L. extract (Vimang). *Eur J Pharmacol* 499:297–305
- Bird CR, Ray JA, Fletcher JD, Boniwell JM, Bird AS (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *Nat Biotechnol* 9:635–638
- Bojórquez G, Gómez Lim MA (1995) Peroxisomal thiolase mRNA is induced during mango fruit ripening. *Plant Mol Biol* 28:811–820
- Bosquet JF, Vegh I, Pouteau-Thouvenot M, Barbier M (1971) Isolement de l'aspergillomarasmine A de cultures de *Colletotrichum gloeosporioides* Penz., agent pathogene des saules. *Ann Phytopath* 3:407–408
- Broekaert WF, Terras FR, Cammue BP, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol* 108:1353–1358
- Carrari F, Fernie AR (2006) Metabolic regulation underlying tomato fruit development. *J Exp Bot* 57:1883–1897
- Campbell MA, Fitzgerald HA, Ronald PC (2002) Engineering pathogen resistance in crop plants. *Transgenic Res* 11:599–613
- Chanliaud E, de Silva J, Strongitharm B, Jeronimidis G, Gidley MJ (2004) Mechanical effects of plant cell wall enzymes on cellulose/xyloglucan composites. *Plant J* 38:27–37
- Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. *Plant Cell* 3:31–40
- Cosgrove DJ, Li LC, Cho HT, Hoffmann-Benning S, Moore RC, Blecker D (2002) The growing world of expansins. *Plant Cell Physiol* 43:1436–1444
- Cruz Hernandez A, Gómez Lim MA (1995) Alternative oxidase from mango (*Mangifera indica* L.) is differentially regulated during fruit ripening. *Planta* 197:569–576
- DeWald SG, Litz RE, Moore GA (1989a) Optimizing somatic embryo production in mango. *J Am Soc Hortic Sci* 114:712–716
- DeWald SG, Litz RE, Moore GA (1989b) Maturation and germination of mango somatic embryos. *J Am Soc Hortic Sci* 114:837–841
- Domínguez M, Vendrell M (1994) Effect of ethylene treatment on ethylene production, EFE activity and ACC levels in peel and pulp of banana fruit. *Postharvest Biol Technol* 4:167–177
- Duwenig E, Steup M, Willmitzer L, Kossmann J (1997) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. *Plant J* 12:323–333
- Engelmann F (1991) In vitro conservation of tropical plant germplasm. *Euphytica* 57:227–243

- Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P (1996) A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. *Plant Physiol* 111:269–273
- Gamborg OL, Miller RA, Ojima K (1968) Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50:151–158
- García D, Escalante M, Delgado R, Ubeira FM, Leiro J (2003a) Anthelmintic and antiallergic activities of *Mangifera indica* L. stem bark components Vimang and mangiferin. *Phytother Res* 17:1203–1208
- García D, Leiro J, Delgado R, Sanmartín ML, Ubeira FM (2003b) *Mangifera indica* L. extract (Vimang) and mangiferin modulate mouse humoral immune responses. *Phytother Res* 17:1182–1187
- Garrido G, Gonzalez D, Lemus Y, García D, Lodeiro L, Quintero G, Delporte C, Nuñez-Selles AJ, Delgado R (2004) In vivo and in vitro anti-inflammatory activity of *Mangifera indica* L. extract (VIMANG). *Pharmacol Res* 50:143–149
- Gavin AL, Conger BV, Trigiano RN (1989) Sexual transmission of somatic embryogenesis in *Dactylis glomerata*. *Plant Breed* 103:251–254
- Giovannoni JJ (2001) Molecular biology of fruit maturation and ripening. *Annu Rev Plant Physiol Plant Mol Biol* 2:725–749
- Giovannoni JJ, DellaPenna D, Bennett AB, Fischer RL (1989) Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. *Plant Cell* 1:53–63
- Gohbara M, Kosuge S, Yamasaki Y, Suzuki A, Tamura S (1978) Isolation, structures and biological activities of colletotrichins, phytotoxic substances from *Colletotrichum nicotianae*. *Agric Biol Chem* 42:1037–1043
- Gómez Lim MA (1997a) Postharvest physiology. In: Litz RE (ed) *The mango – botany, production and uses*. CAB International, Wallingford, Oxon, pp 423–443
- Gómez Lim MA (1997b) Gene isolation in mango fruit: fruit ripening, physiology and molecular biology. *Acta Hort* 455:287–291
- Gómez Lim MA (1998) Physiology and molecular biology of fruit ripening. In: Paredes López O (ed) *Plant biotechnology for food production*. Technomic, London, pp 303–342
- Gómez Lim MA (2001) Genes involved in defence against plant pathogens. In: Valpuesta V (ed.) *Fruit and vegetable biotechnology*. Woodhead, London, pp 114–135
- Gray JE, Picton S, Shabbeer J, Schuch W, Grierson D (1992) Molecular biology of tomato fruit ripening and its manipulation with antisense genes. *Plant Mol Biol* 19:69–87
- Gutiérrez Martínez P, López Gómez R, Gómez Lim MA (2001) Identification of an etr1-homologue from mango expressing during fruit ripening and wounding. *J Plant Physiol* 158:101–108
- Hall LN, Tucker GA, Smith CJS, Watson CF, Seymour GB, Bundick Y, Boniwell JM, Fletcher JD, Ray JA, Schuch W, Bird CR, Grierson D (1993) Antisense inhibition of pectin esterase gene expression in transgenic tomatoes. *Plant J* 3:121–129
- Hammerschlag FA (1992) Somaclonal variation. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford, Oxon, pp 35–55
- Huber DJ (1983) The role of cell wall hydrolases in fruit softening. *Hortic Rev* 5:169–194
- Ishizaki-Nishizawa O, Fujii T, Azuma M, Sekiguchi K, Murata N, Ohtani T, Toguri T (1996) Low-temperature resistance of higher plants is significantly enhanced by a non-specific cyanobacterial desaturase. *Nat Biotechnol* 14:1003–1006
- Iyer CPA, Degani C (1997) Classical breeding and genetics. In: Litz RE (ed) *The mango – botany, production and uses*. CAB International, Wallingford Oxon, pp 49–68
- Jach G, Gornhardt J, Mundy J, Logemann E, Pinsdorf R, Leah A, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *Plant J* 8:97–109
- Jana MM, Nadgauda RS, Rajmohan K, Mascarenhas AF (1994) Rapid somatic embryogenesis from the nucellus of monoembryonic mango varieties. *In Vitro Cell Dev Biol* 30P:55–57

- Jayasankar S, Litz RE (1998) Characterization of embryogenic mango cultures selected for resistance to *Colletotrichum gloeosporioides* culture filtrate and phytotoxin. *Theor Appl Genet* 96:823–831
- Jayasankar S, Litz RE, Schnell RJ, Cruz-Hernandez A (1998) Embryogenic mango cultures selected for resistance to *Colletotrichum gloeosporioides* culture filtrate show variation in random amplified polymorphic DNA (RAPD) markers. *In Vitro Cell Dev Biol-Plant* 34:112–116
- Kalamaki MS, Powell AL, Struijs K, Labavitch JM, Reid DS, Bennett AB (2003) Transgenic over-expression of expansin influences particle size distribution and improves viscosity of tomato juice and paste. *J Agric Food Chem* 51:7465–7471
- Kostermans AFGH, Bompard JM (1993) The mangoes – botany, nomenclature, horticulture and utilization. Academic Press, London
- Kuipers AG, Soppe WJ, Jacobsen E, Visser RG (1994) Field evaluation of transgenic potato plants expressing an antisense granule-bound starch synthase gene: increase of the antisense effect during tuber growth. *Plant Mol Biol* 26:1759–1773
- Kumar S, Patil BC, Sinha SK (1990) Cyanide resistant respiration is involved in temperature rise in ripening mangoes. *Biochem Biophys Res Comm* 168:818–822
- Lad BL, Jayasankar S, Pliego-Alfaro F, Moon PA, Litz RE (1997) Temporal effect of 2,4-D on induction of embryogenic nucellar cultures and somatic embryo development of ‘Carabao’ mango. *In Vitro Cell Dev Biol* 33:253–257
- Lamb CJ, Ryals JA, Ward ER, Dixon RA (1992) Emerging strategies for enhancing crop resistance to microbial pathogens. *Bio/Technology* 10:1436–1445
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197–214
- Leiro J, Garcia D, Arranz JA, Delgado R, Sanmartin ML, Orallo F (2004) An Anacardiaceae preparation reduces the expression of inflammation-related genes in murine macrophages. *Int Immunopharmacol* 4:991–1003
- Litz RE (1984) In vitro somatic embryogenesis from nucellar callus of monoembryonic *Mangifera indica* L. *HortScience* 19:715–717
- Litz RE, Lavi U (1997) Biotechnology. In: Litz RE (ed) The mango – botany, production and uses. CAB International, Wallingford, Oxon, pp 401–423
- Litz RE, Schaffer B (1987) Polyamines in adventitious and somatic embryogenesis in mango (*Mangifera indica* L.). *J Plant Physiol* 128:251–258
- Litz RE, Yurgalevitch C (1997) Effects of 1-aminocyclopropane-1-carboxylic acid, aminoethoxyvinylglycine, methylglyoxal bis-(guanyldiazide) and dicyclohexylammonium sulfate on induction of embryogenic competence of mango nucellar explants. *Plant Cell Tissue Organ Cult* 51:171–176
- Litz RE, Knight RK, Gazit S (1982) Somatic embryos from cultured ovules of polyembryonic *Mangifera indica* L. *Plant Cell Rep* 1:264–266
- Litz RE, Knight RJ, Gazit S (1984) In vitro somatic embryogenesis from *Mangifera indica* L. callus. *Sci Hort* 22:233–240
- Litz RE, Mathews H, Moon PA, Pliego-Alfaro F, Yurgalevitch C, DeWald SG (1993) Somatic embryos of mango (*Mangifera indica* L.). In: Redenbaugh K (ed) Synseeds – applications of synthetic seeds to crop improvement. CRC Press, Boca Raton, pp 409–425
- Litz RE, Moon PA, Monsalud MJ, Jayasankar S, Mathews H (1995) Somatic embryogenesis in *Mangifera indica* L. (mango). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants. Kluwer, Dordrecht, The Netherlands, pp 341–356
- Litz RE, Hendrix RC, Moon PA, Chavez VM (1998) Induction of embryogenic mango cultures as affected by genotype, explanting, 2,4-D and embryogenic nurse culture. *Plant Cell Tissue Org Cult* 53:13–18
- Logemann J, Jach G, Tommerup H, Mundy J, Schell J (1992) Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. *Biotechnology* 10:305–308

- Lu C, Zainal Z, Tucker GA, Lycett GW (2001) Developmental abnormalities and reduced fruit softening in tomato plants expressing an antisense Rab11 GTPase gene. *Plant Cell* 13:1819–1833
- Maheshwari P, Rangaswamy NS (1958) Polyembryony and in vitro culture of embryos of *Citrus* and *Mangifera*. *Ind J Hort* 15:275–282
- Mathews H, Litz RE (1990) Kanamycin sensitivity of mango somatic embryos. *HortScience* 25:965–966
- Mathews H, Litz RE, Wilde DH, Merkel S, Wetzstein HY (1992) Stable integration and expression of  $\beta$ -glucuronidase and NPT II genes in mango somatic embryos. *In Vitro Cell Dev Biol* 28P:172–178
- Mathews H, Litz RE, Wilde DH, Wetzstein HY (1993) Genetic transformation of mango. *Acta Hort* 341:93–97
- Monsalud MJ, Mathews H, Litz RE, Gray DJ (1995) Control of hyperhydricity of mango somatic embryos. *Plant Cell Tissue Organ Cult* 42:195–206
- Mukherjee SK (1950) Mango. Its allopolyploid nature. *Nature* 150:196–197
- Mukherjee SK (1997) Introduction: botany and importance. In: Litz RE (ed.) *The mango – botany, production and uses*. CAB International, Wallingford, Oxon, pp 1–19
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Oeller PW, Wong LM, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* 254:437–439
- Parrisot E (1988) Etude de la croissance rythmique chez de jeunes manguiers (*Mangifera indica* L.). Première partie: description, germination et conservation de graines polyembryonnées de manguiers. *Fruits* 43:97–105
- Pear JR, Sanders RA, Summerfelt KR, Martineau B, Hiatt WR (1993) Simultaneous inhibition of two tomato fruit cell wall hydrolases, pectinmethylesterase and polygalacturonase, with antisense gene constructs. *Antisense Res Dev* 3:181–186
- Peña L, Martín-Trillo M, Juárez J, Pina JA, Navarro L, Martínez-Zapater JM (2001) Constitutive expression of *Arabidopsis* *LEAFY* or *APETALA1* genes in citrus reduces their generation time. *Nat Biotechnol* 19:263–267
- Peñarubia L, Kim R, Giovannoni J, Kim SH, Fischer RL (1992) Production of the sweet protein monellin transgenic plants. *Nat Biotechnol* 10:561–564
- Pliego-Alfaro F, Litz RE, Moon PA, Gray DJ (1995a) Effect of abscisic acid, osmolarity and temperature on in vitro development of recalcitrant mango (*Mangifera indica* L.) nucellar embryos. *Plant Cell Tissue Organ Cult* 44:53–61
- Pliego-Alfaro F, Monsalud MJ, Litz RE, Gray DJ, Moon PA (1995b) Effect of abscisic acid, osmolarity and partial desiccation on the development of recalcitrant mango (*Mangifera indica* L.) somatic embryos. *Plant Cell Tissue Organ Cult* 44:63–70
- Powell AL, Kalamaki MS, Kurien PA, Gurrieri S, Bennett AB (2003) Simultaneous transgenic suppression of LePG and LeExp1 influences fruit texture and juice viscosity in a fresh market tomato variety. *J Agric Food Chem* 51:7450–7455
- Raghuvanshi SS, Srivastava A (1995) Plant regeneration of *Mangifera indica* using liquid shaker to reduce phenolic oxidation. *Plant Cell Tissue Organ Cult* 41:83–85
- Ram S (1997) Propagation. In: Litz RE (ed.) *The mango – botany, production and uses*. CAB International, Wallingford, Oxon, pp 363–400
- Rao AN, Sim YM, Kathagoda N, Hutchinson JF (1982) Cotyledon tissue culture of some tropical fruits. In: Rao AN (ed) *Tissue culture of economically important plants*. COSTED and ANBS, Singapore, pp 124–137
- Rivera Domínguez M, Manzanilla Ramírez MA, Robles González M, Gómez Lim MA (2004) Induction of somatic embryogenesis and plant regeneration of “Ataulfo” mango (*Mangifera indica* L.). *Plant Cell Tissue Organ Cult* 79:101–104
- Rober M, Geider K, Muller-Rober B, Willmitzer L (1996) Synthesis of fructans in tubers of transgenic starch-deficient potato plants does not result in an increased allocation of carbohydrates. *Planta* 199:528–536

- Robson PHR, Smith H (1997) Fundamental and biotechnological applications of phytochrome transgenes. *Plant Cell Environ* 20:831–839
- Robson PHR, McCormac AC, Irvine AS, Smith H (1996) Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene. *Nat Biotechnol* 14:995–998
- Sairam K, Hemalatha S, Kumar A, Srinivasan T, Ganesh J, Shankar M, Venkataraman S (2003) Evaluation of anti-diarrhoeal activity in seed extracts of *Mangifera indica*. *J Ethnopharmacol* 84:11–15
- Seymour GB, Fray RG, Hill P, Tucker GA (1993) Down-regulation of two non-homologous endogenous tomato genes with a single chimaeric sense gene construct. *Plant Mol Biol* 23:1–9
- Sheehy RE, Kramer M, Hiatt WR (1988) Reduction of polygalacturonase activity in tomato fruit by antisense RNA. *Proc Natl Acad Sci USA* 85:8805–8808
- Simon R, Igono MI, Coupland G (1996) Activation of floral meristem identity genes in *Arabidopsis*. *Nature* 384:59–62
- Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* 334:724–726
- Sonnenwald U, Hajirezaei MR, Kossmann J, Heyer A, Trethewey RN, Willmitzer L (1997) Increased potato tuber size resulting from apoplastic expression of a yeast invertase. *Nat Biotechnol* 15:794–797
- Tattersall DB, van Heeswijck R, Hoj PB (1997) Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. *Plant Physiol* 114:759–769
- Tieman DE, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. *Plant Cell* 4:667–679
- Van der Wel H, Arvidson K (1978) Qualitative psychophysical studies on the gustatory effect of the sweet tasting proteins thaumatin and monellin. *Chem Senses Flavor* 3:291–299
- Weigel D, Meyerowitz EM (1994) The ABCs of floral homeotic genes. *Cell* 78:203–209
- Weigel D, Nilsson O (1995) A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377:495–500
- Withers LA (1992) In vitro conservation In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford, Oxon, pp 169–200
- Worrell AC, Bruneau JM, Summerfelt K, Boersig M, Voelker TA (1991) Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. *Plant Cell* 3:1121–1130
- Wu YJ, Huang XL, Xiao JN, Li XJ, Zhou MD, Engelmann F (2003) Cryopreservation of mango (*Mangifera indica* L.) embryogenic cultures. *Cryo Letts* 24:303–314
- Yang Z, Ludders P (1993) Effect of growth regulator and media on in vitro shoot tip culture of different cultivars of mango (*Mangifera indica* L.) rootstocks. *Acta Hort* 341:240–247
- Zainal Z, Tucker GA, Lycett GW (1996) A rab11-like gene is developmentally regulated in ripening mango (*Mangifera indica* L.) fruit. *Biochim Biophys Acta* 1314:187–190
- Zarembinski TI, Theologis A (1994) Ethylene biosynthesis and action: a case of conservation. *Plant Mol Biol* 26:1579–1597
- Zrenner R, Willmitzer L, Sonnewald U (1993) Analysis of the expression of potato uridinediphosphate-glucose pyrophosphorylase and its inhibition by antisense RNA. *Planta* 190:247–252
- Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant J* 7:97–107
- Zrenner R, Schuler K, Sonnewald U (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. *Planta* 198:246–252

## I.4 Papaya

S.D. YEH<sup>1</sup>, H.J. BAU<sup>2</sup>, Y.J. KUNG<sup>1</sup>, and T.A. YU<sup>3</sup>

### 1 Introduction

There are many common names for papaya (*Carica papaya* L.), including papaw or paw paw (Australia), mamao (Brazil) and tree melon (China). The species is believed to be native to southern Mexico and neighboring Central America and to have been taken to Caribbean countries and South-east Asia during the Spanish exploration in the sixteenth century (Storey 1969). It then spread rapidly to India and Africa. Today, it is distributed widely throughout tropical and subtropical regions of the world. A papaya plant has a single, erect, tree-like herbaceous stem, with a crown of large, palmately and deeply lobed leaves. The main stem is cylindrical and hollow, with prominent leaf scars and spongy-fibrous tissue. Leaves are arranged spirally, with petioles extending horizontally up to 1 m long. Trees contain white latex in all their parts. Flowers are male, female, or hermaphrodite, are found on separate trees and are borne in the axils of the leaves. The modified cymose inflorescence allows the flowers to be pollinated easily by wind and insects. The type of flowers produced may change on the same tree, depending on age and environmental factors, such as drought and temperature fluctuations. Hermaphroditic trees consistently produce male flowers, but only with few female flowers that produce fruits during warmer or cooler seasons, whereas female trees are more stable and always produce pistillate flowers under these conditions.

Papaya fruits are fleshy berries and superficially resemble melons. Fruits from female trees are spherical, whereas those from hermaphroditic trees are pyriform, oval or cylindrical with a grooved surface. Since the female fruits contain thinner flesh and more seeds in the central cavity, the hermaphrodite fruits are in more demand by consumers. The fruit is a good source of vitamin A and C (Manshardt 1992). Ripe fruits are largely used as a fresh dessert, while green fruits are often used in salads and pickled or cooked as a vegetable. Papain, a proteolytic enzyme present in the latex, collected mainly from green fruits, has various uses in the beverage, food and pharmaceutical industries,

---

<sup>1</sup> Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan, e-mail: sdteh@nchu.edu.tw

<sup>2</sup> Department of Biotechnology, Transworld Institute of Technology, Yunlin, Taiwan

<sup>3</sup> Department of Biotechnology, Da-Yeh University, Changhua, Taiwan



e.g. chill-proofing beer, tenderizing meat and in drug preparations for digestive ailments (Chan and Tang 1978). It is also used in bathing hides, softening wool and as soap for washing cloth.

Papaya grows relatively easily and quickly from seeds and can reach up to 10 or 12 ft in height. Fruits are ready to be harvested 9–12 months after planting and a tree can continue producing fruits for about 2–3 years, when the height of the plant is too tall for efficient harvesting. Since plant sex cannot be distinguished before flowering, three to five seedlings are normally planted together and only the most vigorous hermaphrodite ones at flowering are selected and cultivated. In 2005, the FAO estimated that about 3.9 hundred thousand hectares and about 6.8 million metric tons of fruit were harvested (Table 1). Brazil, Mexico, Nigeria, India and Indonesia yield more than 70% of the total world production. The extensive adaptation of this plant and wide acceptance of the fruit offer considerable promise for papaya as a commercial crop for local and export purposes. Like banana, pineapple and mango, papaya is one of the important cash crops in the tropics and subtropics. However, the production of this economically important fruit crop is limited by the destructive disease caused by *Papaya ringspot virus* (PRSV), and the fragile and perishable nature of the fruit limit large-scale exportation, with the result that papaya lags behind banana and pineapple in world markets.

**Table 1.** World papaya production in 2005. Data from the Food and Agriculture Organization (FAO), Statistical Division, 2005 (<http://faostat.fao.org/faostat/>)

Country	Hectares (×1000)	Metric tons (×1000)
Brazil	36.5	1650
Mexico	26.3	956
Nigeria	91.0	755
India	80.0	700
Indonesia	24.0	647
Ethiopia	11.0	230
Congo	12.5	210
Peru	12.5	180
China	5.8	160
Venezuela	7.5	140
Colombia	7.0	137
Philippines	5.5	132
Thailand	9.0	131
Cuba	11.0	120
Kenya	8.0	86
Others	41.6	514
Total	389.2	6753

## 2 Worldwide Threat by PRSV Infection

Production of papaya has been limited in many areas of the world due to the disease caused by *Papaya ringspot virus* (PRSV) (Purcifull et al. 1984). Papaya ringspot disease is the major obstacle to large-scale commercial production of papaya (Yeh and Gonsalves 1984). PRSV was first reported in Hawaii in the 1940s (Jensen 1949a), and then became prevalent in Florida (Conover 1964), Caribbean countries (Adsuar 1946; Jensen 1949b), South America (Herold and Weibel 1962), Africa (Lana 1980), India (Capoor and Varma 1948; Singh 1969), the Far East (Wang et al. 1978) and Australia (Thomas and Dodman 1993). To date, most of the major papaya plantation areas of the world suffer from devastation by this virus.

### 2.1 Characteristics of PRSV

PRSV is a member of the genus *Potyvirus* (Purcifull et al. 1984; Murphy et al. 1995), is transmitted non-persistently by aphids and is sap-transmissible in nature. The PRSV genome contains a single-stranded positive sense RNA of about 40 S (De La Rosa and Lastra 1983; Yeh and Gonsalves 1985). Strains of PRSV from Hawaii (Yeh et al. 1992) and Taiwan (Wang and Yeh 1997) have been sequenced; both contain 10,326 nucleotides. The viral RNA encodes a polyprotein that is proteolytically cleaved to generate eight to nine final proteins, including the coat protein for encasidation of the viral genome (Yeh et al. 1992). The virus has a single type of coat protein (CP) of 36 kDa (Purcifull and Hiebert 1979; Gonsalves and Ishii 1980). It induces cylindrical inclusion (CI) (Purcifull and Edwardson 1967) and amorphous inclusion (AI) (Martelli and Russo 1976) in the cytoplasm of host cells. The former consists of a protein of 70 kDa (cylindrical inclusion protein, CIP; Yeh and Gonsalves 1984) and the latter of a protein of 51 kDa (amorphous inclusion protein, AIP; De Mejia et al. 1985a,b). In papaya, PRSV causes severe mosaic and distortion of leaves, ringspots on fruits and water-soaking oily streaks on the upper stems and petioles. It stunts the plant and drastically reduces the fruit size and quality.

### 2.2 No Effective Control Measures

Although tolerant varieties of papaya have been described (Cook and Zettler 1970; Conover 1976; Conover et al. 1986), resistance to PRSV does not exist in *C. papaya*, making conventional breeding difficult (Cook and Zettler 1970; Wang et al. 1978). Tolerance to PRSV has been found in some papaya lines and has been introduced into commercial varieties, but their horticultural properties, such as sweetness, hardness, shape, and shelf-life, are still not commercially desirable (Mekako and Nakasone 1975; Conover and Litz 1978). Other control methods for PRSV, including agricultural practices such as rouging, quarantine, intercropping with corn as a barrier crop and protecting transplanted



seedlings with plastic bags, provide only temporary or partial solutions to the problem (Wang et al. 1987; Yeh and Gonsalves 1994).

PRSV HA 5-1, a cross-protecting mild mutant strain of PRSV that was selected following nitrous-acid treatment of a severe strain (HA) from Hawaii (Yeh and Gonsalves 1984), was tested extensively in the field and has been used commercially in Taiwan and Hawaii since 1985 to permit an economic return from papaya production (Wang et al. 1987; Yeh et al. 1988; Yeh and Gonsalves 1994). However, using the approach involving the deliberate infection of a crop with a mild virus strain to prevent economic damage by more virulent strains has several drawbacks, including the requirement for a large-scale inoculation program, reduction in crop yield and losses of cross-protected plants due to superinfection by virulent strains (Stubbs 1964; Gonsalves and Garnsey 1989; Yeh and Gonsalves 1994).

### 3 A Transgenic Approach for Control of PRSV

The concept of 'pathogen-derived resistance' (Sanford and Johnston 1985) proposes that transforming plants with a pathogen's gene would generate resistance to the infection of the corresponding pathogen. By this concept, Powell-Abel et al. (1986) first demonstrated that transgenic tobacco plants expressing the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) conferred resistance to TMV infection. The CP gene-mediated transgenic resistance has been proven effective for protecting tobacco, tomato, potato and other crops from infection by many different viruses (Beachy 1990; Lomonossoff 1995; Goldbach et al. 2003). Thus, the transgenic approach has become the most effective method to protect crops from virus infection.

In order to solve the problems caused by PRSV, Gonsalves' group at Cornell University and Hawaii started a research project in the late 1980s to develop transgenic papaya. Ling et al. (1991) first demonstrated that the expression of the PRSV HA 5-1 CP gene in tobacco afforded a broad-spectrum of protection against different potyviruses. However, effective gene transfer systems require reliable and efficient procedures for plant regeneration from cells. Fitch and Manshardt (1990) reported that somatic embryogenesis from immature zygotic embryos of papaya could be integrated into a useful gene transfer technology. In the same year, Fitch et al. (1990) incorporated the CP gene of HA 5-1 into papaya via microprojectile bombardment and obtained plants resistant to infection by the severe Hawaii HA strain. Among their transgenic papaya lines, line 55-1 was virtually immune to infection by HA.

#### 3.1 Successful Application of Transgenic Papaya in Hawaii

The plants of transgenic papaya line 55-1 are highly resistant to Hawaiian PRSV isolates under glasshouse and field conditions (Fitch et al. 1992; Lius

et al. 1997). The resistance is triggered by post-transcriptional gene silencing (PTGS), an RNA-mediated specific degradation process of the innate nature of plants against pathogens (Baulcombe 1996, 1999; Hamilton and Baulcombe 1999; Gonsalves 2002). However, the resistance is affected by the sequence identity between the CP transgene and the CP coding region of the challenge virus (Tennant et al. 1994). For example, Rainbow (a CP-hemizygous line derived from SunUp crossed with non-transgenic Kapoho) is susceptible to PRSV isolates outside Hawaii, and SunUp (a CP-homozygous line of 55-1) is resistant to a wider range of isolates from Jamaica and Brazil, but is susceptible to isolates from Thailand and Taiwan (Gonsalves 1998, 2002; Tennant et al. 2001). This characteristic of sequence homology-dependent resistance limits the application of CP-transgenic papaya for controlling PRSV in geographic regions other than Hawaii (Gonsalves 2002).

The field trial of the homozygous line SunUp and hemizygous line Rainbow indicates that both of them offer a good solution to the PRSV problem in Hawaii (Ferreira et al. 2002). By May 1998, Rainbow and SunUp were deregulated by the US Animal and Plant Health Inspection Service and Environmental Protection Agency, and granted approval from the Food and Drug Administration for commercial application (Gonsalves 2002). This is the first successful case of a transgenic fruit tree being commercialized in the world.

### 3.2 Transgenic Papaya in Taiwan

A CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation (Cheng et al. 1996). The transgenic lines obtained showed various levels of resistance, ranging from delay of symptom development to complete immunity (Bau et al. 2003). Several lines highly resistant to the homologous strain (PRSV YK) provide wide-spectrum resistance to three different geographic strains from Hawaii, Thailand and Mexico (Bau et al. 2003). During four repeats of field trials from 1996 to 1999, transgenic papaya exhibited high degrees of protection against PRSV in Taiwan (Bau et al. 2004). Unfortunately, 18 months after plantation in the fourth field trial, unexpected symptoms of severe distortion on fully expanded leaves, stunting of the apex, water-soaking on petioles, and stem and yellow ringspots on fruit were noticed on PRSV CP-transgenic papaya plants. The causal agent was distinguished from PRSV by host reactions and serological properties (Bau 2000) and later identified as *Papaya leaf-distortion mosaic virus* (PLDMV), a potyvirus originating from Okinawa, Japan, in 1954 (Maoka et al. 1996). All of the PRSV CP-transgenic papaya lines were susceptible to PLDMV infection when evaluated under glasshouse conditions. Therefore, in Taiwan, once PRSV CP-transgenic papaya is widely applied for the control of PRSV, PLDMV will become a serious threat to papaya production.

### 3.3 Multiple and Durable Resistance to Different Viruses

In order to control two or more viruses, transgenic plants with multiple resistances have been generated by combining the entire CP gene of more than one virus, with each gene driven by a promoter and a terminator (Fuchs and Gonsalves 1995). Transgenic lines expressing these chimeric CP constructs were resistant to the corresponding viruses and protected from mixed infection, such as *Cucumber mosaic virus*, *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* (Fuchs and Gonsalves 1995; Tricoli et al. 1995; Fuchs et al. 1998). Furthermore, the novel approach proposed by Jan et al. (2000) indicated that transgenic plants with resistance to a potyvirus and a tospovirus could be obtained through the PTGS mechanism by fusing a segment of tospoviral N gene to a segment of potyviral CP gene. The same strategy was used to develop double resistance to both PRSV and PLDMV. An untranslatable chimeric construct that contained the truncated PRSV CP and PLDMV CP genes was then transferred into papaya. Through the PTGS mechanism, transgenic papaya plants carrying this chimeric transgene exhibited resistance to both PRSV and PLDMV under glasshouse conditions (S.D. Yeh, unpublished results). These transgenic papaya plants with a double resistance are believed to have considerable potential for the control of PRSV and PLDMV in Taiwan.

In 4-year field trials, super PRSV strain 5-19 infected transgenic papaya plants were found (Tripathi et al. 2004). The nucleotide identity between the transcript of the CP transgene and PRSV 5-19 RNA is less divergent than that between the CP transgene and other PRSV geographic strains that are not able to overcome the transgenic resistance (Tripathi et al. 2004), indicating that the breakdown of the transgenic resistance is not correlated to the sequence divergence between the infecting virus and the transgene. In order to analyze the role of the gene-silencing suppressor HC-Pro of this super strain, the viral recombinant was constructed by replacing an HC-Pro region of PRSV YK with that of 5-19 and the resistance against the recombinant was evaluated in transgenic papaya. Results showed that the heterologous HC-Pro region of 5-19 alone was sufficient to break down the transgenic resistance in a transgene sequence-homology independent manner, even though the sequences of the transgene transcript shared 100% identity with the genome of the infecting virus (S.D. Yeh, unpublished results). The breakdown of the transgenic resistance by a strong gene-silencing suppressor of a super strain has strong implications for the application of transgenic crops in virus control. We suggest that a chimeric construct targeting multiple viral genes, including the gene determining viral virulence and gene silencing suppression, such as the HC-Pro gene of a potyvirus, may minimize the chance of the emergence of a supervirus that may overcome transgenic resistance.

### 3.4 Transgenic Papaya Generated in Other Geographic Areas

Because of the apparent homology dependence of PRSV CP transgene-associated resistance, the utilization of a CP gene of a local prevalent strain is a prerequisite in order to obtain effective PRSV resistance in transgenic papaya lines for a particular geographic region, as long as genetic variation among virus strains in that region is not a limiting factor (Gonsalves 2002). Using the CP genes of local PRSV isolates to transform local papaya cultivars has been reported in different countries. Lines et al. (2002) used an untranslatable PRSV CP coding region as a transgene to develop two Australian transgenic papaya cultivars which showed immunity to the local PRSV isolate in glasshouse and field tests. Fermin et al. (2004) constructed PRSV-resistant plants by transforming independently with the CP genes of PRSV isolates from two different areas of Venezuela. All the transgenic lines, including  $R_0$  and inter-crossed or self-crossed progenies, revealed different levels of resistance to homologous and heterologous isolates from Hawaii and Thailand. In Florida, transgenic papaya lines carrying the CP gene of the local strain were generated, and the transgenic resistance was introduced to elite papaya cultivars by conventional breeding (Davis and Ying 2004). In addition to the CP gene, the truncated replicase (RP) gene of PRSV was used as a transgene to generate transgenic papaya through *Agrobacterium*-mediated transformation (Chen et al. 2001). PRSV inoculation tests showed that the RP gene conferred resistance to PRSV in transgenic papaya.

## 4 Tissue Culture and Regeneration Techniques

Effective gene transfer systems and the exposure of variation in somaclonal and micropropagation of character-selected parental papaya through tissue culture require readily available explants as well as an efficient system for plant regeneration. Although papaya is not an easy species for regeneration or micropropagation by in vitro techniques, significant progress has been made and provides a foundation for the genetic engineering and micropropagation of this crop.

### 4.1 Embryogenesis and Embryo Rescue Techniques

In the 1970s, embryogenesis in papaya callus was achieved using seeding calli (Arora and Singh 1978) and seedling stem segments (Yie and Liaw 1977). In these studies, it was necessary to transfer calli to another medium for embryogenesis, but the regeneration efficiency was not reported. Other papaya tissues were shown to be able to regenerate into plants, but with low efficiency. Litz et al. (1983) regenerated plants from shoots obtained from papaya cotyledon calli, but did not report on the efficiency of the procedure. Anther culture of

papaya was reported by Tsay and Su (1985). Anthers containing microspores in the tetrad to the early-binucleate stages were successfully cultured for callus initiation and formation. Pollen-derived embryoids and haploid plantlets were obtained. Litz and Conover (1981a, 1982) obtained somatic embryos from the culture of *C. papaya* × *C. cauliflora* hybrid ovules. Manshardt and Wenslaff (1989) showed that hybrid plants of these two species can be obtained from the reciprocal cross *C. cauliflora* × *C. papaya*, with the aid of embryo culture. Somatic embryogenesis and plant regeneration from immature zygotic embryos of *C. papaya* × *C. cauliflora* was described in detail by Chen et al. (1991). These embryo rescue techniques are essential for recovering the interspecific hybrids in which the seeds do not germinate to produce seedlings.

## 4.2 Efficient Regeneration Methods

Fitch and Manshardt (1990) reported that immature zygotic embryos from open-pollinated and self-pollinated *C. papaya* fruits, 90–114 days post-anthesis, produced 2–20 somatic embryos mainly from apical domes. After 6 weeks of culture, about 40–50% of the zygotic embryos became embryogenic, and each embryogenic embryo yielded hundreds of somatic embryos within 5 months of culture on media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). This method is efficient and provides a large quantity of somatic embryos in a relatively short time, and is considered the best system for papaya transformation and regeneration.

Chen et al. (1987) presented results of high-frequency regeneration of papaya plants from root-callus-derived somatic embryos that produced more than 100 plants per explant. Also, high-frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus (Fitch 1993) and petiole explants (Hossain et al. 1993) through organogenesis were reported. The efficient regeneration methods from roots, hypocotyls and petioles also provide a good alternative for later papaya transformation and the regeneration of transgenic plants.

Protoplasts of papaya have been produced in large numbers and calli have been recovered at low frequency (Litz and Conover 1979; Litz 1984). Using highly regenerable suspension cultures of somatic embryos as the source of protoplasts, plant regeneration from protoplasts of the interspecific hybrid of *C. papaya* × *C. cauliflora* was developed by Chen and Chen (1992). However, the regeneration into plants from protoplasts of cultivated papaya species remains difficult. Thus, transformation at the single cell level using papaya protoplast still remains to be developed.

Although somatic embryos derived from immature embryos are most commonly used as material for papaya transformation (Fitch et al. 1990; Cabrera-Ponce et al. 1995, 1996; Cheng et al. 1996; Cai et al. 1999), this method is time-consuming and hindered by seasonal factors. Moreover, the desired sex and other cultivar traits of transgenic lines can be determined only after flow-

ering and fruiting. Using explants obtained from selected sex-type, such as hermaphrodite plants with good horticultural properties, can avoid the disadvantages of immature embryos. We have established an efficient system for inducing adventitious roots from in vitro shoots derived from fruit-bearing field plants (Yu et al. 2000). The adventitious roots can be cultured to induce somatic embryos within 4 months (Lin and Yang 2001). Somatic embryos can be amplified on 2,4-D medium in as large a quantity as those derived from immature embryos, and provide a reliable source for transformation and regeneration.

### 4.3 Effects of Microstatic Agents on Shoot Regeneration

Antibiotics can be added to culture media to control *Agrobacterium* which may affect the plant regeneration process and to select transformants with antibiotic-resistance that is cotransferred with the gene of interest (Shaw et al. 1983). Higher carbenicillin ( $\geq 375 \text{ mg l}^{-1}$ ) promotes callus growth from adventitious root explants, but inhibits somatic embryo formation (Yu et al. 2001). Callus growth is seriously inhibited by the same concentrations of cefotaxime. Somatic embryos derived from neomycin phosphotransferase (*nptII*)-transformed callus are strongly inhibited, with very few somatic embryos developing at kanamycin concentrations of less than  $50 \text{ mg l}^{-1}$ , and no somatic embryos being formed at kanamycin concentration in excess of  $50 \text{ mg l}^{-1}$  (Yu et al. 2003). Thus, during the regeneration process of the transformed cells, the use of appropriate concentrations of carbenicillin and kanamycin at different stages, with their removal at the correct time, are the important parameters for obtaining large numbers of somatic embryos capable of developing into transgenic plantlets.

## 5 Micropropagation Techniques

Papaya is conventionally propagated by seed and, therefore, cultivation is hindered by problems due to the inherent heterozygosity and dioecious nature of this crop (Rajeevan and Pandey 1986). Plants are grown from seeds of open-pollinated flowers and consist of a mixture of genotypes, which exhibit considerable variation in shape, size and flavour of their fruit, and in disease susceptibility (Drew and Smith 1986). There is a need for a rapid clonal propagation protocol to increase the specific sex, tolerant cultivars or virus-resistant transgenic genotypes used in commercial plantations (Lai et al. 2000). Therefore, the use of selected clonal material is imperative in order to obtain uniformity (Teo and Chan 1994). Although conventional techniques of asexual propagation, such as grafting and rooting of cuttings (Allan 1964), are available, they are often tedious and impractical on a large scale (De Winnaar 1988). Thus, propagation by tissue culture is a valuable and reliable approach.



### 5.1 Clonal Propagation from Shoot Tips or Buds

Plants established from shoot tips have been shown to preserve the integrity of the parental genotype (Drew 1996), whereas those regenerated via callus culture may produce genetic off-types (Drew 1988). Methods have been reported in detail for clonal propagation of papaya *in vitro* under different conditions (sex type or season) using shoot tips (Litz and Conover 1981b), shoot tips of young nursery trees (De Winnaar 1988), apical and lateral buds (Drew and Smith 1986), lateral branches of mature field-grown trees (Drew 1988), shoot buds of saplings and fruit-bearing plants (Mondal et al. 1990). These methods are effective for propagating a desired line, but generally do not fulfill the demand of large-scale application. Thus, the rate of shoot-multiplication *in vitro* becomes critical.

### 5.2 In Vitro Preservation and Propagation of Multiple Shoots

Improved techniques for *in vitro* propagation and germplasm storage of papaya were described by Drew (1992). When shoots that developed from axillary buds were dissected and cultured on the rooting medium containing indole-3-butyric acid (IBA) and subsequently transferred to hormone-free medium, the growth rate of explants was reduced significantly by substitution of 1% fructose for 2% sucrose in the medium. After 12 months of incubation at 25 °C without subculture, 100% of the nodal sections produced shoots on the medium containing fructose (Drew 1992). This provides a good method to preserve a selected line and can reduce the cost of frequent subculture. Multiple shoots derived from meristem tips are a good source for large-scale micropropagation. Growth of papaya multishoots can be increased *in vitro* simply by a filter membrane for aeration (Lai et al. 1998), or by controlling ethylene (Lai et al. 2000). Low concentrations of ethylene enhance shoot multiplication immediately after transfer to a new medium, but ethylene becomes detrimental when it accumulates during subsequent culture. Thus, modulation of ethylene during *in vitro* propagation of multishoots is essential to produce large numbers of vigorous shoots that can be easily rooted.

### 5.3 Rooting and Acclimatization

Rooting and acclimatization are critical for establishing papaya plants following micropropagation. Yie and Liaw (1977) noted that rooting and root hair development were enhanced by culturing shoots in medium containing indole-3-acetic acid (IAA). Litz and Conover (1978) reported that  $\alpha$ -naphthaleneacetic acid (NAA) at 5  $\mu$ M and IAA at 0.5–15  $\mu$ M were effective for rooting of the shoots from mature field-grown plants. Drew (1987) noted that an increase in light irradiance and daylength on the foliage enhanced root number and mean root weight per rooted shoot, but light on the basal region of shoots inhibited

root initiation. Also, the addition of riboflavin to the media increased root initiation and root weight, and reduced callus production. Dark incubation reduced callus growth and enhanced the effect of riboflavin on rooting. IBA at 10  $\mu\text{M}$  was optimum for rooting in terms of root number, weight per rooted shoot and quality of the root system. The use of NAA and higher concentrations of IBA resulted in short, thick roots without lateral branches. Shoots derived from mature plants were more difficult to root than those derived from 6-month-old plants. Drew and Miller (1989) observed that decreasing daylength during incubation from 24 to 12 h promoted root initiation; optimum rooting occurred at 27 °C. According to Drew et al. (1993), there are two phases of adventitious root formation: (1) root induction, when exposure to auxin is essential, and (2) root development, when auxin is not required or is inhibitory (Went 1939). Leaves of individual shoots become chlorotic or epinastic and the roots are thick and stumpy when higher IBA concentrations are present for more than 1 week (Drew 1987; Yu et al. 2000). Teo and Chan (1994) noted that papaya microcuttings dipped in IBA and then transferred to water agar produced normal roots without callus forming at basal ends. Drew et al. (1993) discovered that a high proportion of shoots were rooted, and shoot and root growth improved, when shoots were cultured on an IBA-containing medium without riboflavin for 1–3 days and then transferred to an auxin-free medium with riboflavin. Using IBA treatment and vermiculite medium for rooting to achieve high survival rate after acclimatization was described by Manshardt and Drew (1998).

A low-cost protocol was developed to produce high quality root systems for large-scale micropropagation (Yu et al. 2000). Individual shoots (>0.5 cm) with two to three leaves from in vitro-grown papaya multiple shoots are treated with IBA in the dark for 1 week for root induction, and then transferred to vermiculite containing half-strength MS medium (Murashige and Skoog 1962) under aerated conditions for root development. Almost 100% of the plants grow normally and vigorously after glasshouse acclimatization. The main secret of this method is the aeration condition, which avoids ethylene accumulation, considered to be the main cause of weak cultivated shoots which thereby result in poor rooting.

The hermaphrodite-promising plants produced by tissue culture are popular in Taiwan, where more than 80% of papaya plantations (about 2,000 ha) are planted under netting to avoid aphids that can introduce the destructive PRSV. However, the reduction in solar light can elongate the stems and delay the harvest time when papayas are grown under such netting conditions. The characteristics of dwarfing, earlier flowering, and true-type fruits make tissue-culture hermaphrodite plants more acceptable to growers. Although the price for the tissue-culture-derived plants is more than that for seedlings, longer harvest time, consistent quality of fruits and lack of virus infection enable net cultivation to be economically viable.



## 6 Methods of Genetic Transformation

*Agrobacterium*-mediated transformation usually produces transgenic plants with a low copy number, and the transgenes are normally inherited by progeny according to Mendelian segregation as a nuclear trait (Horsch et al. 1984; Budar et al. 1986), although in a few cases non-Mendelian inheritance does occur (Deroles and Gardner 1988). The features of the transgene integration resulting from DNA delivery through microprojectile bombardment often include integration of multiple and full-length transgenes, as well as rearranged copies of the introduced DNA. Multiple transgene insertions are associated with transgene silencing or co-suppression (Jorgensen 1991, 1993). For papaya, despite the fact that *Agrobacterium*-mediated transformation has been proven as an excellent and highly effective system, direct gene delivery through biolistics remains an alternative procedure that is employed by many laboratories.

### 6.1 *Agrobacterium*-Mediated Transformation

*Agrobacterium tumefaciens* is a soil-borne bacterium that, in the presence of a wounded plant, moves towards the host, attaches itself to the wound site, and proceeds to transform the plant cells. The sugars and phenolic compounds exuded by the wounded plant not only signal the pathogenic opportunity to the bacterium, but also induce transcription of the virulence genes. These virulence genes are located on a specific tumor-inducing (Ti) plasmid, which also contains the transferred DNA (T-DNA). Virulence proteins have roles ranging from transcriptional activation to T-DNA processing and export, with certain proteins also having a function in the host. Therefore, *A. tumefaciens* has the capability of inserting the T-DNA into the genome of dicotyledonous plants by its virulence proteins. The use of the *Agrobacterium* system for plant transformation was conceived in 1983 (Chiton 1983; Herrera-Estrella et al. 1983; Hoekema et al. 1983; Schell and Van Montagu 1983).

Even though *Agrobacterium*-mediated transformation was applied to many plants in the mid-1980s, papaya transformation remained difficult. Poor regeneration of transformed cells was one of the limiting factors for papaya genetic engineering. Although plant regeneration from various explants, such as cotyledons (Litz et al. 1983), roots (Chen et al. 1987; Mondal et al. 1994), protoplasts (Chen and Chen 1992), anthers (Tsai and Su 1985) and ovules (Litz and Conover 1982), was attempted, transformed cells were not obtained for regeneration into transgenic plants from these explants. In 1988, transgenic papaya callus was first reported by Pang and Sanford (1988) who used leaf discs, stems and petioles inoculated with *Agrobacterium*, but attempts to regenerate plants from callus were unsuccessful.

As part of the effort to optimize papaya transformation, Fitch and Manshardt (1990) developed protocols for efficient embryogenesis and plant regeneration from immature zygotic embryos and from young hypocotyl sections of papaya

(Fitch 1993). The first successful transformation of papaya by *Agrobacterium* was performed using explants of embryogenic calli and somatic embryos initiated from hypocotyl sections, or immature zygotic embryos (Fitch et al. 1993). However, the plant regeneration process was relatively long (about 13 months) and the transformation rate was also low, with three lines produced from 8 g fresh weight of embryogenic hypocotyl tissues treated, or two lines from 13 g fresh weight of embryogenic tissues derived from hypocotyls and zygotic embryos.

The process of somatic embryo production from petiole explants via an intervening callus stage is also time-consuming (Yang and Ye 1992). After co-cultivation of petioles with *Agrobacterium*, 10–11 months are required for the transformed cells to regenerate into plants (Yang et al. 1996). The long regeneration process and the high rates of production of abnormal transgenic plants, probably due to long exposure to 2,4-D in the medium, limit the application of transformation using petioles. In order to increase the efficiency of transformation, a reliable and efficient method was developed to deliver the CP gene of PRSV YK to papaya by *Agrobacterium* following the liquid-phase wounding treatment of embryogenic tissues with abrasive carborundum powder (Cheng et al. 1996). The transformation rate was 15.9% using the zygotic embryos (52 transgenic somatic embryo clusters out of 327 zygotic embryos treated), which was 10–100 times greater than previous methods. Using this method, a total of 45 putative transgenic lines carrying the PRSV YK cp gene (a Taiwan isolate) were generated, and their resistance to PRSV was evaluated (Bau et al. 2003). It was found that two lines, 18-0-9 and 19-0-1, were immune to PRSV YK and they also provided broad-spectrum resistance to various PRSV HA, TH and MX strains from different geographic areas (Bau et al. 2003).

Based on observations that the viral replicase (RP) gene conferred resistance to virus in other plants (Van Dun et al. 1988; Golemboski et al. 1990), transgenic papaya expressing the RP gene of PRSV was also produced by *Agrobacterium*-mediated gene delivery (Chen et al. 2001). These transgenic plants were shown to be resistant to PRSV. Recently, we produced transgenic papaya carrying the double truncated CP genes of PRSV and PLDMV that displayed high levels of resistance to these two viruses (unpublished results).

Immature embryos are difficult to obtain and often affected by seasonal factors. Because of the polygamous nature of papaya (i.e. with male, female and hermaphroditic plants), the sex types and other horticultural traits of transgenic papaya can only be determined after fruit production. A lengthy breeding procedure is usually required in order to incorporate the desired sex and other useful characters into commercial cultivars of papaya. In view of this, a tissue culture method remains to be developed for high-frequency plant regeneration from desired hermaphroditic plants, coupled with an efficient *Agrobacterium*-mediated transformation approach. This system will be beneficial to the production of commercially desirable papaya genotypes, and would significantly hasten papaya breeding programs.

## 6.2 Direct Gene Transfer by the Biolistic Process

John Sanford at Cornell University, Geneva, New York, invented the particle gun, a device that can literally 'shoot' genetic information obtained from one type of organism into cells of another. This is called the 'biolistic' process or 'microprojectile bombardment'. 'Biolistic' is a short term for 'biological ballistics'; the biolistic process introduces the use of small tungsten particles (microprojectiles) by which biological molecules, such as DNA or RNA, are accelerated at high speed ( $300\text{--}600\text{ m s}^{-1}$ ), by gun powder compressed gas, or other means. A microprojectile can be defined as any small coherent particle capable of being accelerated and then penetrating into cells and tissues. A microprojectile, such as tungsten or gold, should be small enough to enter a cell or tissue in a non-lethal manner, and should be capable of carrying DNA on its surface or in its interior (Klein et al. 1987). The biological molecules are thus driven at high velocity into the plant cell suspensions, callus cultures, meristematic tissues, immature embryos, protocorms, coleoptiles or pollen in a wide range of plants (Southgate et al. 1995). Once inside a cell, the introduced DNA, by some unknown process present in the nuclei, integrates into the plant genome. Using the microprojectile bombardment system, it is possible to transform different plant species, including monocotyledons and conifers that are not susceptible to *Agrobacterium*.

It has been noted that the characteristic features of the transgene integration pattern resulting from microprojectile bombardment include integration of the full length transgene as well as rearranged copies of the introduced DNA. The copy number of both the transgene and rearranged fragments is often highly variable. Most frequently, the multiple transgene copies and rearranged fragments are inherited as a single locus. However, a variable proportion of transgenic events produced by microprojectile bombardment exhibit Mendelian ratios for monogenic and digenic segregation versus events exhibiting segregation distortion. The potential mechanisms underlying these observations have been discussed previously (Pawlowski and Somers 1996). Nevertheless, stable transformation of papaya was achieved using particle bombardment (Fitch et al. 1990). The authors employed three types of embryogenic tissues, including immature zygotic embryos, freshly explanted hypocotyl sections and somatic embryos derived from both these sources, that were bombarded with tungsten particles carrying chimeric *nptII* and *gus* genes. Two years later, the same authors successfully obtained transgenic papaya expressing the CP gene of a PRSV isolate from Hawaii, and one transgenic line (55-1) was shown to be resistant to PRSV (Fitch et al. 1992).

Papaya is susceptible not only to PRSV but also to a range of fungal diseases, including root, stem and fruit rots caused by *Phytophthora palmivora*, which is rampant throughout all papaya growing areas. Zhu et al. (2004) reported the production of transgenic papaya expressing the grapevine stilbene synthase gene VST1 under the control of its own promoter using microprojectile bombardment. These transgenic plants were shown to be more resistant to *P. palmivora*.

## 7 Advances in Molecular Biology

Genes encoding several endopeptidases in papaya latex have been cloned and characterized. Molecular characterization of the enzyme structure and function provides important information for more efficient manipulation and applications of these enzymes in the food and pharmaceutical industries.

### 7.1 Latex Enzymes

The latex of the papaya is well known for being a rich source of the four cysteine endopeptidases, including papain, chymopapain, glycyl endopeptidase and caricain, and other minor constituents. Several cysteine endopeptidases have many applications in the food and pharmaceutical industries. The cDNA sequences of papain (Cohen et al. 1986) and caricain (Revell et al. 1993) and the amino acid sequence of chymopapain (Watson et al. 1990) have been reported. The structure of papain and chymopapain have been determined by X-ray analyses (Drenth et al. 1968; Maes et al. 1996). In addition to industrial and pharmaceutical applications, the protease papain has been shown to protect papaya trees from herbivorous insects (Konno et al. 2004). Future studies on the molecular characterization of the latex enzymes should enable further understanding of their structure and function, leading to novel applications of these enzymes.

### 7.2 Genes Associated with Fruit Ripening

Growth, development and fruit ripening of papaya are regulated by ethylene (Paull and Chen 1983; Lai et al. 2000) which is a gaseous plant hormone. Accumulation of ethylene during post-harvest storage is a key factor hastening fruit ripening, which can result in heavy losses. Cloning and characterization of genes encoding the key enzymes of ethylene biosynthesis are essential for the development of antisense or gene silencing to decrease ethylene production, thereby prolonging the storage and quality of fruits. In papaya, two genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase, which catalyzes the conversion of S-adenosylmethionine to ACC, which is a rate limiting step of ethylene biosynthesis, have been cloned and particularly characterized (Mason and Botella 1997). Moreover, two papaya cDNA sequences for ACC oxidase (CP-ACO1 and CP-ACO2), which catalyzes the conversion of ACC to ethylene, were also isolated and characterized (Lin et al. 1997; Chen et al. 2003). The expression of CP-ACO1 in fruit could be induced before the color break stage, while CP-ACO2 was preferentially expressed at the late stage of ripening (Chen et al. 2003). Apart from the ethylene biosynthetic enzymes, a gene encoding a 45.8-kDa endoxylanase (CpaEXY1), which is correlated with papaya fruit softening, was also cloned and characterized (Chen and Paull 2003). A study of these fruit-ripening-associated genes may lead to a better understanding and control of ripening process in papaya.

### 7.3 Sex-Linked DNA Markers and Sex Determinant

Papaya is a polygamous diploid ( $2n = 18$ ) species with three sex types: male, hermaphrodite and female. Male plants are less useful for economic purposes in the tropics; moreover, in some subtropical areas female plants are less sensitive to temperature fluctuation and fruits are more productive and uniform. Because the flesh is thicker and the cavity is smaller, pyriform fruits from hermaphrodite trees are more desirable for commercial production than the spherical fruits from female plants. Due to the sex preference, either hermaphrodite or female, it is imperative to develop an efficient method for papaya sex identification. Molecular markers tightly linked to the sex of dioecious plants have been reported in several species. These include AFLP (amplified fragment length polymorphism) markers for asparagus (Reamon-Büttner et al. 1998; Reamon-Büttner and Jung 2000) and *Dioscorea tokoro* (Terauchi and Kahl 1999), and RAPD (random amplified polymorphic DNA) markers for *Pistacia vera* (Hormaza et al. 1994) and *Silene dioica* (Di Stilio et al. 1998).

In papaya, RAPD and microsatellite markers linked to sex have been reported (Sondur et al. 1996; Parasnis et al. 1999). RAPD has also been used to determine the sex of dioecious papaya (Deputy et al. 2002; Urasaki et al. 2002). It has been reported that a 450-bp marker fragment, designated PSDM (Papaya Sex Determination Marker), can be detected in all male and hermaphrodite plants but not in the female plants (Urasaki et al. 2002). Based on the PSDM sequences, the two primers, SDP-1 and SDP-2, were synthesized and used as sequence-characterized amplified region (SCAR) markers for DNA amplification. The SCAR primers designed from RAPD markers, SCAR T12 and SCAR W11, specifically for hermaphrodite and male plants showed no recombination in the F2 population. The primer SCAR T12 coupled with SCAR T1, which amplifies a product regardless of sex type, allows a prediction of hermaphrodite plants in a seedling population with an overall accuracy of 99.2% (Deputy et al. 2002). These molecular markers for sex identification of papaya at the seedling stage may facilitate papaya cultivation and breeding by saving time, space and labor cost and avoid growing undesirable plants.

Based on the segregation ratios from crosses among three sex types, Storey (1938) and Hofmeyr (1938) proposed that sex of papaya was determined by a single gene with the three alleles M,  $M^h$  and m. Males (Mm) and hermaphrodites ( $M^h m$ ) are heterozygous and females (mm) are homozygous recessive. MM,  $M^h M^h$  and  $M M^h$  are embryonic lethal (Hofmeyr 1938; Storey 1938), resulting in a 2:1 segregation of hermaphrodites to females from hermaphrodite crossing. The papaya sex locus has been genetically mapped to a specific linkage group (Sondur et al. 1996). Recently, a high-density genetic map of papaya was constructed using 1498 AFLP markers, papaya ringspot virus coat protein insert, morphological sex type and fruit flesh (Ma et al. 2004). These markers were mapped into 12 linkage groups that covered a total length of 3,294 cM, with an average distance of 2.2 cM. The genetic map revealed severe

recombination around the sex determination locus with a total of 225 markers cosegregating with sex types. This high-density genetic map is essential for cloning of specific genes of interest, such as the sex determination gene and other important traits.

Liu et al. (2004) reported that papaya contained a primitive Y chromosome, with a male-specific region that accounted for about 10% of the chromosome but has undergone severe recombination suppression and DNA sequence degeneration. This finding provides direct evidence for the origin of sex chromosomes from autosomes, and also supports the hypothesis that Y chromosome evolution involved at least two steps. First, a single male-sterility mutation led to femaleness and to selection for absence of recombination; second, the Y chromosome evolved as hermaphrodites converted to males by a second mutation or mutations led to female sterility.

## 8 Conclusions

Although papaya is not an easy species for regeneration or micropropagation by in vitro techniques, significant progress has been accomplished and provides a solid foundation for genetic engineering of this crop. Embryogenic tissue derived from premature embryos has been used as the most efficient explant for regeneration of transformed cells into plantlets. Other explants such as root, petiole or hypocotyl have also been used as good alternatives for papaya transformation and regeneration. Culturing of protoplasts and transformation at the single cell level still need more research. Techniques of in vitro preservation and propagation of multiple shoots are mature, and micropropagation can be conducted on a commercial scale as long as the aeration and acclimatization are well controlled. For papaya transformation, both biolistic process and *Agrobacterium*-mediated transformation have been proved to be quite efficient, and transgenic papaya can be efficiently regenerated into plantlets by either process.

The transgenic resistance conferred by the CP gene of a virus has become the most effective method to prevent papaya from infection by the noxious PRSV. In the late 1980s, Gonsalves' group at the Cornell University and University of Hawaii started a program to develop transgenic papaya resistant to PRSV, using the PRSV CP gene, through particle bombardment. In May 1998, PRSV-CP gene transgenic papaya Rainbow and SunUp were deregulated and granted approval for commercialization, representing the first successful application of a transgenic fruit tree in the world. Although the transgenic varieties are not resistant to most other PRSV strains from different geographic areas, breakdown of the resistance in Hawaii has not been recorded. Apart from Hawaii, a CP gene of a native Taiwan strain PRSV YK was used to transform Taiwan papaya cultivars by *Agrobacterium*-mediated transformation. The transgenic lines obtained showed various levels of resistance,



ranging from delay of symptom development to complete immunity. However, during the 4-year-long field trials, the highly resistant PRSV CP-transgenic papaya lines were found to be susceptible to an unrelated potyvirus *Papaya leaf-distortion mosaic virus* (PLDMV) which has been identified in many places in Taiwan. To overcome the potential threat of PLDMV, papaya lines carrying a chimeric transgene, including parts of the CP genes of PRSV and PLDMV, and conferring resistance against both PRSV and PLDMV, were developed. These transgenic papaya lines with double resistance are considered to have great potential for the control of PRSV and PLDMV in Taiwan. During the field test period, a super strain, PRSV 519, able to break down the resistance of YK-CP transgenic lines, was also noticed. The breakdown of the transgenic resistance by 519 is controlled by a strong gene-silencing suppressor, HC-Pro gene, of the virus in a transgene-homology-independent manner. We suggest that a chimeric construct targeting multiple viral genes, such as the genes of the CP, the replicase and the gene silencing suppressor HC-Pro of a potyvirus, may minimize the chances of the emergence of a super virus strain that can overcome the transgenic resistance, and provides more durable resistance against different viruses. Transgenic papaya resistant to pathogens other than PRSV have also been developed. It is expected that resistance to insects, tolerance to herbicides, and other important fruit traits will be the next targets of research.

Advances in understanding the molecular biology of papaya are still at an early stage. Although some genes of latex enzymes and fruit ripening have been reported, molecular analyses related to essential traits such as sweetness, flesh color, hardness, shape and ripening control of fruits remain to be further explored. The sex-linked DNA markers and sex determinant provide a good basis for molecular breeding and for the selection of the desirable hermaphroditic individuals for use in papaya plantations. Further advances in the functional genomic of papaya will surely benefit this unique tropical fruit, one of the shining stars in the world market for fresh fruit.

## References

- Adsuar J (1946) Studies on virus disease of papaya (*Carica papaya*) in Puerto Rico, I. Transmission of papaya mosaic. Puerto Rico Agric Exp Stn Tech Pap 1:1–10
- Allan P (1964) Papaw grown from cuttings. Farming S Afr 39:35–40
- Arora IK, Singh RN (1978) In vitro plant regeneration in papaya. Curr Sci 47:867–868
- Bau HJ (2000) Studies on the resistance of transgenic papaya conferred by the coat protein gene of *Papaya ringspot virus*. PhD Dissertation, National Chung Hsing University, Taichung, Taiwan
- Bau HJ, Cheng YH, Yu TA, Yang JS, Yeh SD (2003) Broad spectrum resistance to different geographic strains of *Papaya ringspot virus* in coat protein gene transgenic papaya. Phytopathology 93:112–120
- Bau HJ, Cheng YH, Yu TA, Yang JS, Liou PC, Hsiao CH, Lin CY, Yeh SD (2004) Field evaluation of transgenic papaya lines carrying the coat protein gene of *Papaya ringspot virus* in Taiwan. Plant Dis 88:594–599
- Baulcombe DC (1996) Mechanisms of pathogen-derived resistance to virus in transgenic plants. Plant Cell 8:1833–1844



- Baulcombe DC (1999) Viruses and gene silencing in plants. *Arch Virol* 15:189–201
- Beachy R (1990) Coat protein-mediated resistance against virus infection. *Annu Rev Phytopathol* 28:451–474
- Budar F, Thia-Toong L, Van Montagu M, Hernalsteens JP (1986) *Agrobacterium*-mediated gene transfer results mainly in transgenic plants transmitting T-DNA as a single Mendelian factor. *Genetics* 114:303–313
- Cabrera-Ponce JS, Vegas-Garcia A, Herrera-Estrella L (1995) Herbicide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. *Plant Cell Rep* 15:1–7
- Cabrera-Ponce JS, Vegas-Garcia A, Herrera-Estrella L (1996) Regeneration of transgenic papaya plants via somatic embryogenesis induced by *Agrobacterium rhizogenes*. *In Vitro Cell Dev Biol-Plant* 32:86–90
- Cai W, Gonsalves C, Tennant P, Fermin G, Souza M, Sarinud N, Jan FJ, Zhu HY, Gonsalves D (1999) A protocol for efficient transformation and regeneration of *Carica Papaya* L. *In Vitro Cell Dev Biol-Plant* 35:61–69
- Capoor SP, Varma PM (1948) A mosaic disease of *Carica papaya* L. in the Bombay province. *Curr Sci* 17:265–266
- Chan HT, Tang CS (1978) The chemistry and biochemistry of papaya. In: Inglett GE, Charolambous G. (eds) *Tropical foods*, vol 1. Academic Press, New York, pp 33–55
- Chen G., Ye CM, Huang JC, Yu M, Li BJ (2001) Cloning of the *Papaya ringspot virus* replicase gene and generation of the PRSV-resistant papaya through the introduction of the PRSV replicase gene. *Plant Cell Rep* 20:272–277
- Chen MH, Chen CC (1992) Plant regeneration from *Carica* protoplasts. *Plant Cell Rep* 11:404–407
- Chen MH, Wang PJ, Maeda E (1987) Somatic embryogenesis and plant regeneration in *Carica papaya* L. tissue culture derived from root explants. *Plant Cell Rep* 6:348–351
- Chen MH, Chen CC, Wang DN, Chen FC (1991) Somatic embryogenesis and plant regeneration from *Carica papaya* × *Carica cauliflora* cultured in vitro. *Can J Bot* 69:1913–1918
- Chen NJ, Paull RE (2003) Endoxylanase expressed during papaya fruit ripening: purification, cloning and characterization. *Funct Plant Biol* 30:433–441
- Cheng YH, Yang JS, Yeh SD (1996) Efficient transformation of papaya by coat protein gene of *Papaya ringspot virus* mediated by *Agrobacterium* following liquid-phase wounding of embryogenic tissues with carborundum. *Plant Cell Rep* 16:127–132
- Chen YT, Lee YR, Yang CY, Wang YT, Yang SF, Shaw JF (2003) A novel papaya ACC oxidase gene (*CP-ACO2*) associated with late stage fruit ripening and leaf senescence. *Plant Sci* 164:531–540
- Chiton MD (1983) A vector for introducing new genes into plants. *Sci Am* 248:36–45
- Cohen LW, Coghlan VM, Dihel LC (1986) Cloning and sequencing of papain-encoding cDNA. *Gene* 48:219–227
- Conover RA (1964) Distortion ringspot, a severe disease of papaya in Florida. *Proc Fla State Hortic Soc* 77:440–444
- Conover RA (1976) A program for development of papaya tolerant to the distortion ringspot virus. *Proc Fla State Hortic Soc* 89:229–231
- Conover RA, Litz RE (1978) Progress in breeding papaya with tolerance to *Papaya ringspot virus*. *Proc Fla State Hortic Soc* 91:182–184
- Conover RA, Litz RE, Malo SE (1986) ‘Cariflora’ – a *Papaya ringspot virus*-tolerant papaya for south Florida and the Caribbean. *HortScience* 21:1072
- Cook AA, Zettler FW (1970) Susceptibility of papaya cultivars to papaya ringspot and papaya mosaic virus. *Plant Dis Rep* 54:893–895
- Davis MJ, Ying Z (2004) Development of papaya breeding lines with transgenic resistance to *Papaya ringspot virus*. *Plant Dis* 88:352–358
- De La Rosa M, Lastra R (1983) Purification and partial characterization of papaya ringspot virus. *Phytopathol Z* 106:329–336
- De Mejia MVG, Hiebert E, Purcifull DE (1985a) Isolation and partial characterization of the amorphous cytoplasmic inclusions protein associated with infections caused by two potyviruses. *Virology* 142:24–33

- De Mejia MVG, Hiebert E, Purcifull DE, Thornbury DW, Pirone TP (1985b) Identification of potyviral amorphous inclusion protein as a nonstructural, virus-specific protein related to helper component. *Virology* 142:34–43
- Deputy JC, Ming R, Ma H, Liu Z (2002) Molecular markers for sex determination in papaya (*Carica papaya* L.). *Theor Appl Genet* 106:107–111
- Deroles SC, Gardner RC (1988) Expression and inheritance of kanamycin resistance in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. *Plant Mol Biol* 11:355–364
- De Wijnnaar W (1988) Clonal propagation of papaya in vitro. *Plant Cell Tissue Organ Cult* 12:305–310
- Di Stilio VS, Kesseli RV, Mulcahy DL (1998) A pseudoautosomal random amplified polymorphic DNA marker for the sex chromosomes of *Silene dioica*. *Genetics* 149:2057–2062
- Drenth J, Jansonius JN, Koekoek R, Swen HM, Wolthers BG (1968) Structure of papain. *Nature* 218:929–932
- Drew RA (1987) The effects of medium composition and cultural conditions on in vitro root initiation and growth of papaya (*Carica papaya* L.). *J Hort Sci* 62:551–556
- Drew RA (1988) Rapid clonal propagation of papaya in vitro from mature field grown trees. *HortScience* 23:609–611
- Drew RA (1992) Improved techniques for in vitro propagation and germplasm storage of papaya. *HortScience* 27:1111–1124
- Drew RA (1996) An improved in vitro technique for in vitro clonal propagation of fruit species. *Proc Int Symp on Tropical Fruits*, Kuala Lumpur, pp 35–40
- Drew RA, Miller RM (1989) Nutritional and cultural factors affecting rooting of papaya (*Carica papaya* L.) in vitro. *J Am Soc Hort Sci* 64:767–773
- Drew RA, Smith NG (1986) Growth of apical and lateral buds of papaya (*Carica papaya* L.) as affected by nutritional and hormonal factors. *J Hort Sci* 61:535–543
- Drew RA, McComb JA, Considine JA (1993) Rhizogenesis and root growth of *Carica papaya* L. in vitro in relation to auxin sensitive phase and use of riboflavin. *Plant Cell Tissue Organ Cult* 33:1–7
- Fermin G., Inglessis V, Garboza C, Rangle S, Dagert M, Gonsalves D (2004) Engineered resistance against *Papaya ringspot virus* in Venezuelan transgenic papaya. *Plant Dis* 88:516–522
- Ferreria SA, Pitz KY, Manshardt R, Zee F, Fitch M (2002) Virus coat protein transgenic papaya provides practical control of *Papaya ringspot virus* in Hawaii. *Plant Dis* 86:101–105
- Fitch MMM (1993) High frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus. *Plant Cell Tissue Organ Cult* 32:205–212
- Fitch MMM, Manshardt RM (1990) Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). *Plant Cell Rep* 9:320–324
- Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment. *Plant Cell Rep* 9:189–194
- Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992) Virus resistance papaya plants derived from tissues bombarded with the coat protein gene of *Papaya ringspot virus*. *Bio/Technology* 10:1466–1472
- Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL (1993) Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos. *Plant Cell Rep* 12:245–249
- Fuchs M, Gonsalves D (1995) Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of Zucchini mosaic virus and Watermelon mosaic virus 2 to mixed infections by both potyviruses. *Bio/Technology* 13:1466–1473
- Fuchs M, Tricoli DM, Carney KJ, Schesser M, McPerson JR, Gonsalves D (1998) Comparative virus resistance and fruit yield of transgenic squash with single and multiple coat protein genes. *Plant Dis* 82:1350–1356
- Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. *Virus Res* 92:207–212

- Golemboski DB, Lomonosoff GP, Zaitlin M (1990) Plants transformed with a *Tobacco mosaic virus* nonstructural gene sequence are resistant to the virus. *Proc Natl Acad Sci USA* 87:6311–6315
- Gonsalves D (1998) Control of *Papaya ringspot virus* in papaya: a case study. *Annu Rev Phytopathol* 36:415–437
- Gonsalves D (2002) Coat protein transgenic papaya “acquired” immunity for controlling papaya ringspot virus. *Curr Top Microbiol Immunol* 266:73–83
- Gonsalves D, Garnsey SM (1989) Cross-protection techniques for control of plant virus diseases in the tropics. *Plant Dis* 73:592–597
- Gonsalves D, Ishii M (1980) Purification and serology of papaya ringspot virus. *Phytopathology* 70:1028–1032
- Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286:950–952
- Herold F, Weibel J (1962) Electron microscopic demonstration of papaya ringspot virus. *Virology* 18:307–311
- Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* 303:209–213
- Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179–180
- Hofmeyr JDJ (1938) Genetical studies of *Carica papaya* L. *S Afr J Sci* 35:300–304
- Hormaza JI, Dollo L, Polito VS (1994) Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. *Theor Appl Genet* 89:9–13
- Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A (1984) Inheritance of functional foreign genes in plants. *Science* 223:496–498
- Hossain M, Rahman SM, Islam R, Joarder OI (1993) High efficiency plant regeneration from petiole explants of *Carica papaya* L. through organogenesis. *Plant Cell Rep* 13:99–102
- Jan FJ, Fagoaga C, Pang SZ, Gonsalves D (2000) A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *J Gen Virol* 81:2103–2109
- Jensen DD (1949a) Papaya virus diseases with special reference to papaya ringspot. *Phytopathology* 39:191–211
- Jensen DD (1949b) *Papaya ringspot virus* and its insect vector relationship. *Phytopathology* 39:212–220
- Jorgensen R (1991) Silencing of plant genes by homologous transgenes. *AgBiotech News Inf* 4:265N–273N.
- Jorgensen R (1993) The germinal inheritance of epigenetic information in plants. *Trans R Soc Lond B* 339:173–181
- Klein RM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Bio/Technology* 24:384–386
- Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, Kohno K (2004) Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. *Plant J* 37:370–378
- Lai CC, Yu TA, Yeh SD, Yang JS (1998) Enhancement of in vitro growth of papaya multishoots by aeration. *Plant Cell Tissue Organ Cult* 53:221–225
- Lai CC, Yeh SD, Yang JS (2000) Enhancement of papaya axillary shoot proliferation in vitro by controlling the available ethylene. *Bot Bull Acad Sin* 41:203–212
- Lana AF (1980) Transmission and properties of virus isolated from *Carica papaya* in Nigeria. *J Hort Sci* 55:191–197
- Lin CM, Yang JS (2001) Papaya somatic embryo induction from fruiting-bearing field plants: effects of root supporting material and position of the rooting explants. *Acta Hort* 560:489–492
- Lin CT, Lin MT, Shaw JF (1997) Cloning and characterization of a cDNA for 1-aminocyclopropane-1 carboxylate oxidase from papaya fruit. *J Agric Food Chem* 45:526–530

- Lines RE, Persley D, Dale JL, Drew R, Bateson MF (2002) Genetically engineered immunity to *Papaya ringspot virus* in Australian papaya cultivars. *Mol Breed* 10:119–129
- Ling K, Namba S, Gonsalves C, Slightom JL, Gonsalves D (1991) Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the *Papaya ringspot virus* coat protein gene. *Bio/Technology* 9:752–758
- Litz RE (1984) Papaya. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) *Handbook of plant cell culture*. Vol. 2. Macmillan, New York, pp 349–368
- Litz RE, Conover RA (1978) In vitro propagation of papaya. *HortScience* 13:241–242
- Litz RE, Conover RA (1979) Development of systems for obtaining parasexual *Carica* hybrids. *Proc Fla State Hortic Soc* 92:180–182
- Litz RE, Conover RA (1981a) In vitro polyembryony in *Carica papaya* L. ovules. *Z Pflanzenphysiol* 104:285–288
- Litz RE, Conover RA (1981b) Effect of sex type, season, and other factors on in vitro establishment and culture of *Carica papaya* L. explants. *J Am Soc Hort Sci* 106:792–794
- Litz RE, Conover RA (1982) In vitro somatic embryogenesis and plant regeneration from *Carica papaya* L. ovular callus. *Plant Sci Lett* 26:153–158
- Litz RE, O'Hair SK, Conover RA (1983) In vitro growth of *Carica papaya* L. cotyledons. *Sci Hort* 19:287–293
- Liu Z, Moore PH, Ma H, Ackerman CM (2004) A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature* 427:348–352
- Lius S, Manshardt RM, Fitch MMM, Slightom JL, Sanford JC, Gonsalves D (1997) Pathogen-derived resistance provides papaya with effective protection against *Papaya ringspot virus*. *Mol Breed* 3:161–168
- Lomonosoff GP (1995) Pathogen-derived resistance to plant viruses. *Ann Rev Phytopathol* 33:323–343
- Ma H, Moore PH, Liu Z, Kim MS, Yu Q (2004) High-density linkage mapping revealed suppression of recombination at the sex determination locus in papaya. *Genetics* 166:419–436
- Maes D, Bouckaert J, Poortmans F, Wyns L, Looze Y (1996) Structure of chymopapain at 1.7 Å resolution. *Biochemistry* 35:16292–16298
- Manshardt RM (1992) Papaya. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford, Oxon, pp 489–511
- Manshardt RM, Drew RA (1998) Biotechnology of papaya. *Acta Hort* 461:65–73
- Manshardt RM, Wenslaff TF (1989) Zygotic polyembryogenesis and plant regeneration in interspecific hybrids of *Carica papaya* and *C. cauliflora*. *J Am Soc Hort Sci* 114:684–689
- Maoka T, Kashiwazaki S, Tsuda S, Usugi T, Hibino H (1996) Nucleotide sequence of the capsid protein gene of the *Papaya leaf-distortion mosaic potyvirus*. *Arch Virol* 141:197–204
- Martelli GP, Russo M (1976) Unusual cytoplasmic inclusions induced by *Watermelon mosaic virus*. *Virology* 72:352–362
- Mason MG, Botella JR (1997) Identification and characterisation of two 1-aminocyclopropane-1-carboxylate (ACC) synthase cDNAs expressed during papaya (*Carica papaya*) fruit ripening. *Aust J Plant Physiol* 24:239–244
- Mekako HU, Nakasone HY (1975) Interspecific hybridization among 6 *Carica* species. *J Am Soc Hort Sci* 100:237–242
- Mondal M, Gupta S, Mukherjee BB (1990) In vitro propagation of shoot buds of *Carica papaya* L. (Caricaceae) var. Honey Dew. *Plant Cell Rep* 8:609–612
- Mondal M, Gupta S, Mukherjee BB (1994) Callus culture and plantlet production in *Carica papaya* (var. Honey Dew). *Plant Cell Rep* 13:390–393
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summer MD (1995) *Virus taxonomy*. Springer, Vienna
- Pang SZ, Sanford JC (1988) *Agrobacterium*-mediated gene transfer in papaya. *J Am Soc Hort Sci* 113:287–291

- Parasnis AS, Ramakrishna W, Chowdari KV, Gupta VS, Ranjekar PK (1999) Microsatellite (GATA) *n* reveals sex-specific differences in papaya. *Theor Appl Genet* 99:1047–1052
- Paull RE, Chen NJ (1983) Postharvest variation in cell wall degrading enzymes of papaya (*Carica papaya*) during ripening. *Plant Physiol* 72:382–385
- Pawlowski WP, Somers DA (1996) Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Mol Biotechnol* 6:17–30
- Purcifull DE, Edwardson JR (1967) *Watermelon mosaic virus*: tubular inclusion in pumpkin leaves and aggregates in leaf extracts. *Virology* 32:393–401
- Purcifull DE, Hiebert E (1979) Serological distinction of *Watermelon mosaic virus* isolates. *Phytopathology* 69:112–116
- Purcifull DE, Edwardson JR, Hiebert E, Gonsalves D (1984) *Papaya ringspot virus*. CMI/AAB Description of Plant Viruses, No. 292. Association of Applied Biologists, Warwick
- Powell-Abel P, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the *Tobacco mosaic virus* coat protein gene. *Science* 232:738–743
- Rajeevan MS, Pandey RM (1986) Lateral bud culture of papaya (*Carica papaya* L.) for clonal propagation. *Plant Cell Tissue Organ Cult* 5:181–188
- Reamon-Büttner SM, Jung C (2000) AFLP-derived STS markers for the identification of sex in *Asparagus officinalis* L. *Theor Appl Genet* 100:432–438
- Reamon-Büttner SM, Schondelmaier J, Jung C (1998) AFLP markers tightly linked to the sex locus in *Asparagus officinalis* L. *Mol Breed* 4:91–98
- Revell DF, Cummings NJ, Baker KC, Collins ME, Taylor MA, Sumner IG, Pickersgill RW, Conner-ton IF, Goodenough PW (1993) Nucleotide sequence and expression in *Escherichia coli* of cDNAs encoding papaya proteinase omega from *Carica papaya*. *Gene* 127:221–225
- Sanford JC, Johnston SA (1985) The concept of parasite-derived resistance: deriving resistance gene from the parasite's own genome. *J Theor Biol* 113:395–405
- Schell J, Van Montagu M (1983) The Ti plasmids as natural and as practical gene vectors for plants. *Bio/Technology* 1:175–180
- Shaw CH, Leemans J, Van Montagu M, Schell J (1983) A general method for the transfer of cloned genes to plant cell. *Gene* 23:315–330
- Singh AB (1969) A new virus disease of *Carica papaya* in India. *Plant Dis Rep* 53:267–269
- Sondur SN, Manshardt RM, Stiles JI (1996) A genetic linkage map of papaya based on randomly amplified polymorphic DNA markers. *Theor Appl Genet* 93:547–553
- Southgate EM, Davey MR, Power JB, Marchant R (1995) Factors affecting the genetic engineering of plants by microprojectile bombardment. *Biotechnol Adv* 13:631–651
- Storey WB (1938) Segregation of sex types in Solo papaya and their application to the selection of seed. *Am Soc Hort Sci Proc* 35:83–85
- Storey WB (1969) Papaya (*Carica papaya* L.). In: Ferwerda FP, Wit F (eds) Outline of perennial crop breeding in the tropics. H. Veenman en Zonen BV, Wageningen, pp 389–407
- Stubbs LL (1964) Transmission and protective inoculation studies with viruses of the citrus tristeza complex. *Aust J Agr Res* 15:752–770
- Tennant P, Fermin G, Fitch MMM, Manshardt R, Slightom JL, Gonsalves D (2001) *Papaya ringspot virus* resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Eur J Plant Pathol* 107:645–653
- Tennant PF, Gonsalves C, Ling KS, Fitch M, Manshardt R, Slightom JL, Gonsalves D (1994) Differential protection against *Papaya ringspot virus* isolates in coat protein gene transgenic papaya and classically cross-protected papaya. *Phytopathology* 84:1359–1366
- Teo CKH, Chan LK (1994) The effects of agar content, nutrient concentration, genotype and light intensity on the in vitro rooting of papaya microcutting. *J Hort Sci* 62:267–273
- Terauchi R, Kahl G (1999) Mapping of the *Dioscorea tokoro* genome: AFLP markers linked to sex. *Genome* 42:752–762
- Thomas JE, Dodman RL (1993) The first record of *Papaya ringspot virus*-type P in Australia. *Aust Plant Pathol* 22:2–7

- Tricoli DM, Carney KJ, Pussell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to *Cucumber mosaic virus*, *Watermelon mosaic virus 2*, and *Zucchini yellow mosaic virus*. *Bio/Technology* 13:1458–1465
- Tripathi S, Bau HJ, Chen LF, Yeh SD (2004) The ability of *Papaya ringspot virus* strains overcoming the transgenic resistance of papaya conferred by the coat protein gene is not correlated with higher degrees of sequence divergence from the transgene. *Eur J Plant Pathol* 110:871–882
- Tsay HS, Su CY (1985) Anther culture of papaya (*Carica papaya* L.). *Plant Cell Rep* 4:28–30
- Urasaki N, Tokumoto M, Tarora K, Ban Y, Kayano T, Tanaka H, Oku H, Chinen I, Terauchi R (2002) A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.). *Theor Appl Genet* 104:281–285
- Van Dun CMP, Van Vloten-Doting L, Bol JF (1988) Expression of *Alfalfa mosaic virus* cDNA 1 and 2 in transgenic tobacco plants. *Virology* 163:572–578
- Wang CH, Yeh SD (1997) Divergence and conservation of the genomic RNAs of Taiwan and Hawaii strains of *Papaya ringspot virus*. *Arch Virol* 142:271–285
- Wang HL, Wang CC, Chiu RJ, Sun MH (1978) Preliminary study on *Papaya ringspot virus* in Taiwan. *Plant Prot Bull* 20:133–140
- Wang HL, Yeh SD, Chiu RJ, Gonsalves D (1987) Effectiveness of cross protection by mild mutants of papaya ringspot virus for control of ringspot disease of papaya in Taiwan. *Plant Dis* 71:491–497
- Watson DC, Yaguchi M, Lynn KR (1990) The amino acid sequence of chymopapain from *Carica papaya*. *Biochem J* 266:75–81
- Went FW (1939) The dual effect of auxin on root formation. *Am J Bot* 26:24–29
- Yang JS, Ye CA (1992) Plant regeneration from petioles of in vitro regenerated papaya (*Carica papaya* L.) shoots. *Bot Bull Acad Sin* 33:375–381
- Yang JS, Yu TA, Cheng YH, Yeh SD (1996) Transgenic papaya plants from *Agrobacterium*-mediated transformation of petioles of in vitro propagated multishoots. *Plant Cell Rep* 15:549–564
- Yeh SD, Gonsalves D (1984) Evaluation of induced mutants of *Papaya ringspot virus* for control by cross protection. *Phytopathology* 74:1086–1091
- Yeh SD, Gonsalves D (1985) Translation of papaya ringspot virus RNA in vitro: detection of a possible polyprotein that is processed for capsid protein, cylindrical-inclusion protein, and amorphous-inclusion protein. *Virology* 143:260–271
- Yeh SD, Gonsalves D (1994) Practices and perspective of control of papaya ringspot virus by cross protection. *Adv Dis Vector Res* 10:237–257
- Yeh SD, Gonsalves D, Wang HL, Namba R, Chiu RJ (1988) Control of *Papaya ringspot virus* by cross protection. *Plant Dis* 72:375–380
- Yeh SD, Jan FJ, Chiang CH, Doong TJ, Chen MJ, Chung PH, Bau HJ (1992) Complete nucleotide sequence and genetic organization of *Papaya ringspot virus* RNA. *J Gen Virol* 73:2531–2541
- Yie ST, Liaw SI (1977) Plant regeneration from shoot tips and callus of papaya. *In Vitro* 13:564–567
- Yu TA, Yeh SD, Cheng YH, Yang JS (2000) Efficient rooting establishment of papaya plantlets by micropropagation. *Plant Cell Tissue Organ Cult* 61:29–35
- Yu TA, Yeh SD, Yang JS (2001) Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. *Bot Bull Acad Sin* 42:281–286
- Yu TA, Yeh SD, Yang JS (2003) Comparison of the effects of kanamycin and geneticin on regeneration of papaya from root tissue. *Plant Cell Tissue Organ Cult* 74:169–178
- Zhu YJ, Agbayani R, Jackson MC, Tang CS, Moore PH (2004) Expression of the grapevine stilbene synthase gene VST1 in papaya provides increased resistance against diseases caused by *Phytophthora palmivora*. *Planta* 220:241–250



## I.5 Pineapple

M.R. DAVEY<sup>1</sup>, S. SRIPAORAYA<sup>2</sup>, P. ANTHONY<sup>1</sup>, K.C. LOWE<sup>3</sup>, and J.B. POWER<sup>1</sup>

### 1 Introduction

Pineapple is economically the most important member of the family Bromeliaceae and follows banana, mangoes and citrus in terms of world tropical fruit production. The crop is amenable to cultivation on large-scale plantations and, as a consequence, is grown in at least 26 countries, Thailand, the Philippines, Brazil, China and India being the leading producers. The former two countries dominate world production (Rohrbach et al. 2003). In 2004, world pineapple production was 15,288,018 metric tons with a yield of 181,303 hectogram ha<sup>-1</sup> (<http://faostat.fao.org>). Fresh pineapple is important in both domestic and export markets. The edible portion of the fruit, which constitutes about 60% of the weight, contains approximately 85% water, 0.4% protein, 14% sugar (sucrose), 0.1% fat and 0.5% fibre (Rangan 1984). The fruit is rich in vitamins A and B, while the juice has 75–83% sucrose and 7–9% citric acid on a dry weight basis. Canned fruit contributes most to world trade in this crop, with recent increases in the production of concentrated juice and chilled cut fruit (Hepton and Hodgson 2003). Pineapple is exploited in several other ways. The waste from fruit processing is used as animal feed, while pineapple is the source of the complex proteolytic enzyme bromelain of importance in the pharmaceutical market and as a meat tenderising agent. Fibres from leaves yield a fine fabric, pina cloth. Some chimeric forms of pineapple are marketed as ornamental plants. Although terrestrial in habit, pineapple, as an herbaceous monocotyledonous perennial, also exhibits some features of epiphytes in storing small quantities of water in its leaves and their axils. Bartholomew et al. (2003) edited an excellent treatise on pineapple which discusses aspects of its morphology, anatomy, physiology, pests and diseases, post-harvest handling and storage.

<sup>1</sup> Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK, e-mail: [mike.davey@nottingham.ac.uk](mailto:mike.davey@nottingham.ac.uk)

<sup>2</sup> Faculty of Agriculture Nakhonsithammarat, Rajamangala University of Technology Srivijaya, Tungyai, Nakhonsithammarat 80240, Thailand

<sup>3</sup> School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, UK



## 2 Taxonomy and Breeding of Pineapple

The taxonomy of pineapple has been reviewed by Coppens d'Eekenbrugge and Leal (2003). As these authors emphasise, pineapple classification has been complex and inconsistent, which led them to examine in detail the origin and evolution of this plant and to propose a simple and more consistent classification. They regrouped pineapples into the single genus *Ananas*, characterised by the unique feature of the inflorescence, which is fused into a multiple fruit or syncarp. The genus *Ananas* contains two species, namely *macrodon* and *comosus*, with the species proposed earlier by Smith and Downs (1979) being down-graded to five varieties, namely *ananassoides*, *bracteatus*, *comosus*, *erectifolius* and *paraguazensis*. Such a classification also eliminates the five groups, namely Cayenne, Queen, Spanish, Pernambuco (or Abacaxis) and Perolera, based on horticultural characteristics (Py et al. 1987).

All of the varieties (var.) of *A. comosus* have a diploid chromosome complement of 50 very small chromosomes, although triploid, tetraploid and heteroploid plants also exist. *A. macrodon* is a natural tetraploid with 100 chromosomes (Collins 1960); *A. comosus* var. *comosus* is highly variable and is the main pineapple of cultivation because of its large fruit. Pineapple cvs. must be highly self-sterile to be commercially viable. Thus, most fail to set seed when grown in isolation, but may produce seed when cultivated with other cultivars (cvs.), as in germplasm collections. The greatest genetic diversity in wild pineapples is exhibited by *A. comosus* var. *ananassoides*, with tolerance to wilt, nematodes, crown and root rots. Other wild pineapples show resistance to heart rot and fuscariosis, with some being tolerant to drought and flooding. The breeding system of pineapple combines vegetative reproduction with allogamous sexual reproduction, the latter providing most of the genetic variation. Chan et al. (2003) discussed aspects of the cytogenetics and breeding of pineapple, the generation of progeny and their agronomic potential.

Following the initiation of breeding programmes in Hawaii in 1914, similar hybridisation projects have been instigated in other countries, including Taiwan, Malaysia, the Philippines, Côte d'Ivoire, Cuba, Puerto Rico, Australia and Brazil. The widely grown cv. Smooth Cayenne has been one of the favoured parents because of its adaptability and reliable fruit quality, both as fresh produce and following processing. Breeders have given priority to obtaining new cvs. that are spineless, coupled with other traits such as resistance to diseases, pests, heart and root rots, excellent flavour, early ripening and well-shaped fruits with a crisp non-fibrous texture and a golden-yellow colour. However, in spite of considerable long-term efforts, new hybrids have not surpassed Smooth Cayenne in their performance. In general, the narrow genetic base of cultivated pineapples is a cause for concern.

Chan et al. (2003) highlighted the potential application of biotechnological approaches, such as exposure of somaclonal variation and the introduction of specific characteristics by transformation, in attempts to expand the genetic base of pineapple. Such characteristics include resistance to black-heart disease

and mealybug wilt, inhibition of precocious flowering and extending the shelf-life of harvested fruit. They proposed that genetic manipulation will be the tool to fine-tune the performance of current cvs., but emphasise that this approach must be supported by adequate gene mapping of pineapple chromosomes.

### **3 Genetic Relationships in Pineapple: Application of Biochemical and Molecular Markers**

Clarification of genetic diversity in pineapple is essential to facilitate the development of breeding strategies and to maximise output from hybridisation programmes. Isozyme polymorphism was first employed in attempts to clarify the genetic relationships of some cvs. (Noyer et al. 1996). For example, de Wald et al. (1988a, 1992) used starch gel electrophoresis of leaf samples and reported that two peroxidases and three phosphoglucosmutases could be used to identify 15 of the 27 cvs. which they examined. Subsequently, Aradhya et al. (1994) evaluated isozyme variation in 161 accessions of pineapple. These authors concluded that *A. comosus* contained five genetically diverse groups, but, interestingly, these groups did not match perfectly with the traditional varietal groupings based, at that time, on morphological characteristics. They proposed that differentiation in pineapple may result from ecological isolation, rather than genuine genetic divergence. More extensive studies of isozymes revealed 31 alleles at 10 loci (Duval et al. 2001).

DNA restriction fragment length polymorphism (RFLP) has been more efficient in revealing genetic variation, Noyer (1991) making the first RFLP analysis in pineapple using five heterologous and nine homologous probes on 35 accessions. However, poor variability was found with only three polymorphic markers of 14 used in the investigation. Later, Ruas et al. (1995) used random amplified polymorphic DNA (RAPD) analysis to estimate the genetic relationships of a limited number of pineapple cvs. and concluded that the cvs. Smooth Cayenne and Primavera were closely related. In addition, Noyer et al. (1996) analysed RFLPs for ribosomal DNA variability in 92 clones representative of the principal genetic groups of pineapple from the French CIRAD-FLHOR collection in the French West Indies (Martinique). Using eight restriction endonucleases, the hybridisation probe TA71 and a fragment of rDNA from wheat, these authors concluded the genus *Ananas* to be very homogeneous, as based on rDNA analyses.

Later, Duval et al. (2001) reported a comprehensive investigation involving 18 homologous genetic probes and 301 accessions of the genus *Ananas*. Most of the plants were collected in Brazil, French Guiana, Venezuela and Paraguay and maintained in field collections at CIRAD-FLHOR or the Brazilian Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA)-Mandioca e Fruticultura Institutes. Other accessions were from the USDA National Clonal Germplasm Repository in Hawaii. Most variation was found in wild germplasm, with less

variability in cultivated pineapples. Interestingly, this result confirmed the first RFLP observations of Noyer (1991). As Duval et al. (2001) emphasised, such a difference in polymorphism between cultivated plants and their wild relatives is remarkable in a plant which, predominately, is propagated vegetatively, supporting the hypothesis of the role of sexual reproduction in the wild in the genus *Ananas*, even if it is observed only rarely (Duval et al. 1997). Again, cvs. grown for their fruit were relatively homogeneous, in spite of their wide morphological variation, with clones of the ubiquitous cv. Smooth Cayenne grouping together, whether from Guiana or from other localities. These data, indicating low molecular diversity, suggest a unique origin for each of these cvs., followed by clonal selection for agronomically useful traits (Duval et al. 2001).

In another report of the same year, RAPD analysis was applied to eight commercial pineapple cvs. grown in Thailand, two sexual hybrids and two wild pineapples, to determine whether DNA analyses correlated with morphology (Sripaoraya et al. 2001a). DNA amplification products were compared from 16 arbitrary 10-mer primers to enable the construction of a dendrogram. The molecular analyses confirmed morphological groupings for seven of the eight commercial cvs. Overall, RAPD analysis, combined with results of the investigation of Duval et al. (2001), should assist in selecting those germplasms most suitable for future breeding programmes. Nevertheless, there are pineapple cvs. whose pedigrees are unclear, while others that are genetically identical have been given different names.

#### **4 Biochemical and Molecular Approaches to Flowering and Fruit Production in Pineapple**

Pineapple is a non-climacteric fruit, since it does not have an autocatalytic burst of ethylene during ripening. Nevertheless, ethylene is involved in the ripening process. Ethylene and ethylene-releasing compounds induce flowering in the Bromeliaceae, although the response is not rapid in pineapple. In pineapple, the endogenous production of ethylene is based on the methionine cycle. Specifically, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase converts methionine to ACC which, in turn, is converted by ACC oxidase to ethylene (McKeon et al. 1995). Pineapple is a short-day plant, but in regions where it is cultivated, the photoperiod is usually too long and temperatures above the optimum for fruit production. Consequently, the natural flowering of pineapple is unpredictable, emphasising the need to stimulate uniform flowering in plantations. Ethylene or ethephon are examples of flower-inducing compounds, while the application of ACC to shoots also induces flowering in adult bromeliads. The latter observation has prompted studies of ACC synthase in pineapple. Oeller and Gutterson (1997) used a PCR-based approach to define the ACC synthase gene that is normally expressed in pineapple fruits,

particularly ovary tissues. Using sequence information compiled from the databases for a number of known genes, these authors synthesised primers to amplify five products from pineapple genomic DNA. Expression analyses using RT-PCR and RNase protection experiments revealed four of the five genes to be expressed in pineapple. In leaves, two genes were found to be wound-inducible, a third gene was auxin-induced, while a fourth gene was expressed constitutively in young plants.

Cazzonelli et al. (1998) targeted pineapple as a model to clone and to characterise two ripening-inducible cDNAs coding for ACC synthase (*acacc-1*) and ACC oxidase (*acaco-1*) of the ethylene biosynthetic pathway. A model protocol was optimised to extract high quality RNA from fruit tissues, this being required because of the acidity and high polyphenol content of such material. *Acacc-1* was found to be a 1,080 bp cDNA fragment encoding 360 amino acids, including 10 of 12 amino acid residues conserved in all aminotransferases. Southern analysis suggested the presence of only a single copy of *acacc-1* in the pineapple genome. *Acaco-1* was a partial-length cDNA clone of 611 bp which coded for 203 amino acids representing approximately 66% of the ACC oxidase open reading frame, with the presence of one or two copies of the gene in the pineapple genome. Northern analysis showed the expression of *acaco-1* to be highly induced in wounded leaves and, to a lesser extent, in tissues of ripening fruits.

A retrotransposon was first detected in the genome of pineapple by Thomson et al. (1998). The sequence from a 2.6 kb cloned fragment of this element had greatest similarity to the *dell Lilium henryi* retrotransposon and the *gypsy*/Ty3 group of retroelements. The order of the genes from 5' to 3' was reverse transcriptase, ribonuclease H and integrase. The integrase domain contained the amino acid sequence motifs, which have been associated with recognition of the long terminal repeats and with the cutting/joining reactions required for integration of similar retroelements into the host genome. The retrotransposon exists as a population of variable sequences which are dispersed throughout the pineapple genome, with Southern hybridisation showing that the retrotransposon integrated repeatedly into the pineapple genome. The reading frame of the element was not interrupted by stop codons, suggesting that it is still potentially capable of transposing.

In a recent investigation to gain a more comprehensive understanding of the molecular basis of pineapple fruit development, Moyle et al. (2005) surveyed a range of expressed sequences from green unripe and yellow ripe fruit tissues. An abundant metallothionein transcript was estimated to account for up to 50% of all expressed sequence tag (EST) library clones. Genes that were identified in green fruit included a bromelain and a bromelain inhibitor, while those in yellow fruit libraries included a MADS box gene, together with genes for protein synthesis, including homologues of ribosomal L10 and the translation factors SUI1 and eIF5A. Libraries for both green unripe and yellow ripe fruits contained clones associated with response to oxidative stress and the detoxification of free radicals. Improved knowledge of the molecular biology

of pineapple could facilitate in decreasing the time plants exhibit a 'ripeness to respond' and, consequently, reducing the cost of fruit production. Such background information also provides a baseline for constructing genes of agronomic potential for use in the genetic manipulation of this crop.

## 5 Tissue Culture-Based Technologies for Pineapple Improvement

Pineapple is propagated vegetatively in the field from crowns, slips, suckers (ground shoots) or hapas (base of the fruit slips), with each of these vegetative propagules requiring a different period from planting to flowering and fruit harvest. Generally, this is about 13–15 months for suckers and 16–18 months for crowns. Both slips and hapas are produced in some cvs., although others may fail to produce these structures. Several difficulties are associated with vegetative propagation. For example, the rate of production of propagules by crowns, slips or suckers is slow and the propagules are limited in number. In addition, the crowns of the plants are sold attached to the fresh fruit and, therefore, are not available as propagules. The latter may also be limited because of the plant genotype. For example, both smooth and spiny cvs. may produce relatively few suckers when, for commercial reasons, planting densities are high ( $6\text{--}8 \times 10^4$  plants  $\text{ha}^{-1}$ ). Traditional methods usually produce up to 10 plants from one single parental plant per annum, although this may increase tenfold in some cases. The availability of propagules, which is often restricted, is generally regarded as a key factor in limiting the expansion of commercial pineapple production.

Micropropagation by tissue culture offers a reliable alternative to conventional methods of plant multiplication, not only for mass production per se, but also, more importantly, to clone elite plants with desirable fruit characteristics. Additionally, virus-free plants can be generated through meristem culture. Desirable genes can also be transferred into pineapple cvs. and species via *Agrobacterium*-mediated DNA delivery and/or particle bombardment into totipotent tissues.

### 5.1 Micropropagation of Pineapple

Rapid multiplication of elite germplasm through tissue culture-based procedures is advantageous when large populations of plants are required to meet plantation demands. More than four decades have passed since cultures for the micropropagation of pineapple were established by Aghion and Beauchesne (1960). Mapes (1973) produced numerous plants and protocorm-like structures from shoot tips of the cv. Cayenne in shaken liquid Murashige and Skoog (MS) (1962)-based medium supplemented with  $30 \text{ mg l}^{-1}$  adenosine. Later, Lakshmi Sita et al. (1974) introduced meristems from slips into culture, while Mathews et al. (1976) and Mathews and Rangan (1979) cultured terminal

and axillary buds from crowns of an unspecified cv., again on MS medium but supplemented with  $1.8 \text{ mg l}^{-1}$  naphthaleneacetic acid (NAA),  $2.0 \text{ mg l}^{-1}$  indole-3-butyric acid (IBA) and  $2.0 \text{ mg l}^{-1}$  kinetin. Shoots developed roots on MS medium containing only NAA ( $0.1 \text{ mg l}^{-1}$ ) as the growth regulator, with plants being recovered within 6–8 weeks of the initiation of the cultures. This cv. was also cloned by maintaining axillary buds excised from crowns on MS-based medium with 25% (v:v) coconut milk, followed by transfer to half-strength MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  benzyladenine (BA). A single crown produced about 5,000 new plants within 12 months (Zepeda and Sagawa 1981).

Other workers also investigated the behaviour of lateral and axillary buds in vitro (Cabral et al. 1984). Simultaneously, Rangan (1984) cultured lateral buds from crowns on MS-based medium with  $1.8 \text{ mg l}^{-1}$  NAA,  $2.0 \text{ mg l}^{-1}$  IBA and  $2.0 \text{ mg l}^{-1}$  kinetin to induce multiple bud formation, these concentrations of growth regulators being similar to those employed earlier (Mathews and Rangan 1979). The regenerated shoots developed roots when  $0.17 \text{ mg l}^{-1}$  NAA was included in the medium in the presence of  $0.39 \text{ mg l}^{-1}$  IBA. Later, de Wald et al. (1988b) determined the efficiency of culture for rapid propagation of the cvs. Cayenne, Red Spanish and Perolera; axillary buds were maintained on MS medium supplemented with  $102 \text{ mg l}^{-1}$  inositol,  $0.4 \text{ mg l}^{-1}$  thiamine HCl,  $2.0 \text{ mg l}^{-1}$  NAA and  $2.0 \text{ mg l}^{-1}$  BA at  $24\text{--}27^\circ\text{C}$  with a 16-h photoperiod. Proliferating explants were multiplied in shaken liquid medium of the same composition with subculture every 28 days. Plants, each about 2.5 cm in height, were transferred to compost in individual pots, enclosed in plastic bags and incubated in a growth chamber with suitable light at a temperature of  $28^\circ\text{C}$ . In all cvs., regenerated plants more than 3.0 cm in height had a survival rate of 100% following transfer to compost. However, as expected, differences in culture responses were noted between genotypes, with the cv. Cayenne producing less shoots during each subculture compared to the cvs. Red Spanish and Perolera.

Liu et al. (1989) produced 40,000 plants of the cv. Red Spanish by culturing meristems on MS-based medium containing  $0.1 \text{ mg l}^{-1}$  2,4-dichlorophenoxyacetic acid (2,4-D) and  $0.5 \text{ mg l}^{-1}$  BA. Cote et al. (1991) also used axillary buds from crowns on MS-based medium with the same concentration of BA and  $0.2 \text{ mg l}^{-1}$  indole-3-acetic acid (IAA), followed by MS medium in which BA was retained but IAA was omitted. Dissecting each plant into two or four pieces at subculture increased their rate of multiplication. Later, Moore et al. (1992) established cultures from axillary buds excised from crowns on MS medium, but with sucrose reduced from 3% to 0.3% (w/v), in the presence of 0.8% (w/v) Difco Bacto agar,  $102 \text{ mg l}^{-1}$  inositol,  $0.40 \text{ mg l}^{-1}$  thiamine HCl,  $2.0 \text{ mg l}^{-1}$  NAA and  $2.0 \text{ mg l}^{-1}$  BA. The authors multiplied the regenerated shoots in liquid medium of the same composition. In the case of the cv. Kew, shoot apices multiplied within 7 days of culture initiation on MS medium with  $0.02 \text{ mg l}^{-1}$  NAA, bud proliferation being stimulated by replacing NAA with  $2.25 \text{ mg l}^{-1}$  benzylaminopurine (BAP; synonym BA) and  $0.17 \text{ mg l}^{-1}$  IAA.



Shoot buds regenerated into plants following their transfer to MS medium with  $0.02 \text{ mg l}^{-1}$  NAA (Hirimburegama and Wijesinghe 1992). Fitchet (1990) and Fitchet-Purnell (1993) also produced plants by culturing lateral buds removed from pineapple crowns on semi-solid Murashige and Tucker (MT) (1969) medium containing NAA, IAA and kinetin, each at  $2.0 \text{ mg l}^{-1}$ . Subsequently, regenerated shoots were micropropagated in liquid MT medium with  $2.0 \text{ mg l}^{-1}$  of both NAA and kinetin; shoots were rooted in MT medium with the same concentrations of NAA and  $500 \text{ mg l}^{-1}$  malt extract. Rooted plants were immersed overnight in a solution of  $2.0 \text{ g l}^{-1}$  benomyl to prevent fungal attack, before being established in compost.

Lateral buds were also used as the experimental material by Almeida et al. (1996) to optimise the BAP concentration and, in turn, to maximise shoot regeneration in the cvs. Primavera and Perola. Explants were transferred to MS medium containing  $2.0 \text{ mg l}^{-1}$  IAA and  $40 \text{ g l}^{-1}$  sucrose with a range of BAP concentrations. Regenerated shoots were transferred to MS medium with  $40 \text{ g l}^{-1}$  sucrose,  $7.0 \text{ g l}^{-1}$  agar and different concentrations of BAP, to stimulate their proliferation. Thereafter, shoots were subcultured to medium lacking growth regulators for root induction. The optimum multiplication rates for the cv. Perola were obtained with  $2.0$  or  $1.0 \text{ mg l}^{-1}$  BAP for the establishment and proliferation stages, while for the cv. Primavera, the optimum concentrations of BAP were  $3.0$  and  $2.0 \text{ mg l}^{-1}$ , respectively.

An interesting *in vitro* procedure involving etiolated nodal explants was reported for the cv. Cayenne (Kiss et al. 1995). Shoot etiolation was induced by placing decapitated plants on MS medium with  $1.86 \text{ mg l}^{-1}$  NAA, prior to incubation in the dark at  $28^\circ\text{C}$  for 30–40 days. Subsequently, etiolated shoots were transferred to N6 medium (Chu 1978) with  $5.3 \text{ mg l}^{-1}$  kinetin or  $4.5 \text{ mg l}^{-1}$  BA. Shoots regenerated from the nodes of stems within 4–6 weeks of culture, with several thousand plants being established within 12 months. Leaf bases or shoot base explants were used by Teng (1997) to produce nodular tissues when the components of MS medium were reduced to half strength in the presence of  $1.0 \text{ mg l}^{-1}$  NAA and  $1.0 \text{ mg l}^{-1}$  BA. The tissues proliferated when dissected into pieces, each about 1–3 mm in size, and regenerated shoots when the concentrations of NAA and BA were reduced by tenfold. Regenerated shoots rooted in the same medium, with batches of plants being produced every 6 weeks using this protocol. The regenerative capacity of nodular tissues was greater than from explants by direct regeneration through organogenesis, or by a callus phase.

Attention has been focused on the micropropagation of pineapple cvs. grown in Bangladesh (Khatun et al. 1997), especially the cvs. Giant Kew and Khulna Local (Rahman et al. 2001), and those cultivated in Thailand. In the latter case, Sriporaya et al. (2003) introduced into culture axillary and terminal buds from suckers of the Thai cv. Phuket using MT medium containing  $2.0 \text{ mg l}^{-1}$  IBA,  $2.0 \text{ mg l}^{-1}$  NAA and  $2.0 \text{ mg l}^{-1}$  kinetin as growth regulators. Each original explant produced 10–20 shoots within 35 days of culture initiation, although about 5–8% of these micropropagated shoots were phenotypic variants with



albino striped leaves. Such variant shoots were discarded. Phenotypically normal shoots were transferred to MS-based medium with  $2.0 \text{ mg l}^{-1}$  BA, with subculture every 35–42 days, to provide a continuous supply of axenic shoots for further experimentation and/or transfer to the glasshouse. It was possible, using the simple protocol described, to generate 21,000 plants within 12 months from one axillary or terminal bud, although the regenerants needed to be screened for their phenotypes and any morphologically abnormal plants eliminated. Nevertheless, this number of plants was considerably more than could be produced using protocols from earlier reports. For example, Zepeda and Sagawa (1981) generated about 5,000 plants in the same period using MS medium with 25% (v/v) coconut milk, followed by culture on half-strength MS medium containing  $1.0 \text{ mg l}^{-1}$  BA. The simple micropropagation procedure developed by Sripaoraya et al. (2003) was extended to other Thai cvs., namely Intrachitdang, Intrachitkao, Naglae, Pattavia, Petburi No. 2, Sawee, Tainan, Tradsithong and hybrid germplasms. Phenotypically normal plants developed roots on agar-solidified MS medium lacking growth regulators and were established *ex vitro* in the glasshouse.

Importantly, the cost-efficiency of micropropagating pineapple has been improved by exploiting bioreactor technology involving temporary immersion of plant tissues in liquid medium. For example, Escalona et al. (1999) connected a culture vessel to a reservoir of liquid medium. Compressed air forced the medium to flow from the reservoir to immerse the plants. Reversal of the air-flow withdrew the medium from the plant culture container; electronic timers controlled the frequency and length of the immersion period. In studies of the use of temporary immersion compared to the use of semi-solid and static liquid media, temporary immersion increased the multiplication rate and biomass over a 42-day period. In addition, these workers assessed temporary immersion combined with paclobutrazol as a medium supplement, this compound promoting the formation of clusters of compact buds and limiting leaf development. Optimum multiplication occurred when explants were cultured in MS-based medium with  $2.1 \text{ mg l}^{-1}$  BA,  $0.3 \text{ mg l}^{-1}$  NAA and  $1 \text{ mg l}^{-1}$  paclobutrazol, the most uniform plants ( $191.8 \text{ plants l}^{-1}$  of medium) being generated after 4 weeks of culture in the presence of paclobutrazol. These results demonstrated the positive effect of paclobutrazol on pineapple micropropagation, enabling confirmation of the earlier observations of these workers (Escalona et al. 1995). The temporary immersion system also reduced, by 20%, the unit production cost for pineapple plants compared to conventional methods of multiplication (Escalona et al. 1999).

In a more recent report describing the use of bioreactors, Firoozabady and Gutterson (2003) compared air lift, rotating and periodic (temporary) immersion vessels, the most successful being the latter in terms of biomass production. Their simple bioreactor was based on a 10-L Nalgene vessel to mass propagate the cv. Smooth Cayenne. Longitudinal sections or the bases of leaves from *in vitro* grown shoots were used to initiate the cultures, with plant material being immersed in liquid medium for  $5\text{--}10 \text{ min h}^{-1}$ . The medium was

based on the MS formulation prepared to half strength with the addition of B5 vitamins (Gamborg et al. 1968),  $30 \text{ g l}^{-1}$  sucrose,  $1.5 \text{ mg l}^{-1}$  BA and  $0.5 \text{ mg l}^{-1}$  NAA. Explants were cultured for 4 weeks, followed by transfer to medium of similar composition, but with  $1.0 \text{ mg l}^{-1}$  BA and  $0.4 \text{ mg l}^{-1}$  gibberellic acid (GA3). Shoots were harvested after 8 weeks in the bioreactor. Regenerated shoots were rooted in trays containing a shallow layer of liquid medium with  $0.5 \text{ mg l}^{-1}$  BA and the same concentration of NAA. Using this procedure, 6,000–8000 shoots were generated from two parental shoots within 6 months. The clonal fidelity of regenerated plants was assessed in plantations in Costa Rica and Indonesia. Variation, other than extent of spination, was not observed and, importantly, fruits were unaffected by the culture parameters.

Hamasaki et al. (2005) showed that 8 mM glutamine increased shoot regeneration by organogenesis from leaf bases of the cv. Smooth Cayenne from 46 to 70%, with an increase in shoot vigour. The induction of shoot buds occurred within the first 7 days of culture in glutamine-supplemented medium, with an associated increase in the concentration of endogenous IAA and a 50% increase in the concentration of the cytokinin isopentenyladenine in cultured explants. It will be interesting to determine the effect of glutamine in the medium with explants cultured in temporary immersion bioreactors of the type described by Firoozabady and Gutterson (2003).

Temporary immersion bioreactors have been developed by other research groups to facilitate physiological investigations of pineapple tissues. For example, Gonzalez-Olmedo et al. (2005) used such bioreactors to control the change from photomixotrophic to photoautotrophic growth of pineapple plantlets. The effects of light were evaluated by photosynthetic photon flux density, sucrose concentration and carbon dioxide evolution within the culture vessels. The authors demonstrated that light was the parameter that exerted the most influence on plant quality.

In commercial laboratories, every effort must be made to minimise plant production costs, often by modification or elimination of standard procedures. In this respect, Teixeira et al. (2006) developed a protocol to sterilise culture media by the addition of sodium hypochlorite, eliminating the requirement for autoclaving. These authors reported that chlorine concentrations equal to or greater than 0.0003% resulted in effective sterilisation of the medium, with more than a two-fold increase in biomass and number of shoots of pineapple compared to the test system during the experimental period. Be and Debergh (2006) also addressed production costs and devised a low-cost procedure to micropropagate pineapple. Following multiplication on MS-based medium with  $0.8\text{--}1.0 \text{ mg l}^{-1}$  BA, shoot proliferation and rooting were carried out in a nethouse rather than a growth room, with nethouse-grown plants being about 20% less costly to produce than those from growth chambers. Effort has also been focused on improving protocols to maximise normal plant development during culture and to minimise plant damage and stress during acclimation to *ex vitro* conditions. As a baseline for these investigations, Barboza et al. (2006) studied the anatomical features of micropropagated plants and found

that whilst the basic structure of the pineapple leaf did not change under *in vitro* conditions, phenotypic plasticity during this period was expressed through changes in the frequency of stomata, cuticular and epidermal wall thickenings, the sinuosity of the cell walls of aquiferous parenchyma and the presence of papillary cells.

Whilst attention has focused on the micropropagation of plants of edible pineapples, some investigations have been directed to ornamental pineapples. For example, *Neoregelia cruenta*, which is endemic to eastern Brazil, is used extensively in landscape gardening because of its attractive red-tipped leaves. Carneiro et al. (1999) developed an efficient plant regeneration system for the induction of shoots from the bases of leaf explants derived from seedlings. Shoot regeneration was influenced by seedling age and the combination of growth regulators in the medium. Most shoot regeneration was obtained from leaf explants excised from 7-week-old axenically grown seedlings, followed by culture on MS medium containing  $4.9 \text{ mg l}^{-1}$  BA and  $0.5 \text{ mg l}^{-1}$  NAA. However, the conversion of shoots to plants was most efficient in those regenerants that developed on MS medium with BA at  $1.0 \text{ mg l}^{-1}$  combined with  $0.5 \text{ mg l}^{-1}$  NAA. *Aechmea fasciata* is another ornamental pineapple which has been micropropagated (Jones and Murashige 1974; Vinterhalter and Vinterhalter 1994; Carneiro et al. 1999), the inclusion of a cytokinin (BA) in the medium being essential for adventitious shoot induction.

## 5.2 Callus Induction from Cultured Explants

Whilst micropropagation of pineapple has involved direct shoot regeneration from cultured explants, callus has also been induced from explants prior to plant regeneration. For example, Mathews and Rangan (1981) established callus from basal regions of *in vitro* grown shoot buds of an unnamed market cv. cultured on filter paper discs in contact with MS medium supplemented with  $400 \text{ mg l}^{-1}$  casein hydrolysate, 15% (v/v) coconut milk and  $10 \text{ mg l}^{-1}$  NAA. Such callus produced shoot buds after transfer to MS medium lacking NAA and with coconut milk reduced to 5% (v/v). The callus-derived buds, in turn, produced multiple shoots when subcultured onto MS medium with  $1.8 \text{ mg l}^{-1}$  NAA,  $2.0 \text{ mg l}^{-1}$  IBA and  $2.1 \text{ mg l}^{-1}$  kinetin. Regenerated shoots were induced to root on medium prepared according to the formulation of White (1963), with  $0.05 \text{ mg l}^{-1}$  NAA and  $0.4 \text{ mg l}^{-1}$  IBA as growth regulators. Subsequently, rooted plants were transferred to soil.

Other protocols for shoot regeneration were also reported by Rangan (1984), who initiated multiple shoots from lateral buds using MS medium with  $1.8 \text{ mg l}^{-1}$  NAA,  $2.0 \text{ mg l}^{-1}$  IBA and  $2.1 \text{ mg l}^{-1}$  kinetin. Subsequently, such shoots produced callus at their bases on transfer to MS medium supplemented with  $5.39 \text{ mg l}^{-1}$  NAA,  $5.2 \text{ mg l}^{-1}$  IAA and  $2.1 \text{ mg l}^{-1}$  kinetin. This callus was maintained by regular subculture on MS medium, in which the NAA concentration was increased to  $10.61 \text{ mg l}^{-1}$ , with 15% (v/v) coconut milk and  $400 \text{ mg l}^{-1}$

casein hydrolysate. Shoots were induced on the callus using MS medium with the concentration of coconut milk reduced to 5% (v/v), whilst maintaining casein hydrolysate at  $400 \text{ mg l}^{-1}$ . The regenerated shoots developed roots on transfer to White's medium with  $0.05 \text{ mg l}^{-1}$  NAA and  $0.38 \text{ mg l}^{-1}$  IBA. Leaf explants, excised from in vitro grown plants, also developed callus capable of shoot regeneration (Mathews and Rangan 1979). However, it was not clear from the latter studies whether these regenerated plants produced fruit.

It is interesting to note that MS-based medium has been employed in most tissue culture investigations with pineapple. Thus, Rosa-Marquez and Lizardi (1987) and Liu et al. (1989) reported the use of MS medium, in this case supplemented with  $10 \text{ mg l}^{-1}$  BAP and  $4 \text{ mg l}^{-1}$  NAA, to induce the formation of callus in the dark from the cv. Red Spanish. Callus transferred to MS medium with  $4 \text{ mg l}^{-1}$  NAA regenerated shoots after 2–3 weeks of incubation under diffuse light at  $26^\circ\text{C}$ . In a paper published in the same year, Wakasa (1989) cultured syncarp tissues of the cv. Cayenne on MS medium with  $10 \text{ mg l}^{-1}$  of both NAA and BA. Fitchet (1990) also induced callus from apical crown regions of the cv. Queen, but on MT medium containing  $40 \text{ mg l}^{-1}$  NAA, 15% (v/v) coconut milk and  $400 \text{ mg l}^{-1}$  casein hydrolysate. This author observed that callus was not capable of differentiation unless the cells passed through a stage where their pigmentation changed from yellow to green. Developing shoot buds could be seen at the periphery of such callus, as well as within the tissue.

The commercial importance of pineapple cultivation in Thailand has stimulated investigations with the cv. Phuket, in which the bases of leaves excised from micropropagated shoots (leaves 4–7 from the shoot apex) produced callus within 14 days on MS medium containing several combinations of 2,4-D and BA, with  $0.5 \text{ mg l}^{-1}$  2,4-D and  $2.0 \text{ mg l}^{-1}$  BA inducing 83% of the leaf bases to undergo callus formation and shoot regeneration. The presence of 2,4-D in the medium was crucial for this morphogenic response; all the plants regenerated from leaf bases were phenotypically true-to-type. It was estimated that about 59,000 plants could be regenerated from one original leaf explant in a 12-month period, with rooted plants being produced within 90 days of culture initiation (Sripaoraya et al. 2003).

As already indicated, the fruit of pineapple is generally seedless because of strong self-incompatibility. However, natural or artificial cross pollination may occur between *A. comosus* var. *comosus* and *A. comosus* var. *ananassoides*, and the var. *comosus* with *A. comosus* var. *bracteatus*, resulting in  $F_1$  seed production. Such seeds have been used as source material for callus initiation. Other examples have been cited. Following sexual hybridisation of the cvs. Giant Kew and Queen, hybrid embryos were cultured on MS medium with  $0.1 \text{ mg l}^{-1}$  BA (Srinivasa Rao et al. 1981). Callus formation commenced from the radicles and eventually enveloped the embryos, with continuously growing callus being established by repeated subculture of this primary tissue. Subsequently, shoot buds differentiated from the callus and, once separated, were established on MS-based medium. On average, 20–25 plants were obtained from callus initiated from a single hybrid zygotic embryo. Benega et al. (1996a) also investigated

seed germination *in vitro*, with callus initiation from hybrid embryos being achieved on MS medium with  $1.0 \text{ mg l}^{-1}$  NAA. Such callus regenerated plants on medium prepared to the same formulation, but with  $0.3 \text{ mg l}^{-1}$  NAA and  $2.1 \text{ mg l}^{-1}$  BA. However, the establishment of plants *ex vitro* was not confirmed. Benega et al. (1996b) also cultured unfertilised ovules, collected 5–10 days after anthesis, from the pineapple cvs. Serrana, Smooth Cayenne, Pina Blanca, Red Spanish and Perolera, most ovules of the cvs. Serrana, Smooth Cayenne and Red Spanish producing callus on MS medium containing 3 or 4% (w/v) sucrose and a 5:1 combination of 3,6-dichloro-2-methoxybenzoic acid (dicamba) with BAP. However, the precise concentrations of the growth regulators used in these experiments were not given.

### 5.3 Somatic Embryogenesis in Cultured Tissues of Pineapple

Although shoot regeneration in cultured tissues of pineapple occurs predominantly by organogenesis, conditions have been reported that result in plant regeneration via somatic embryogenesis. In the first documentation of this shoot regeneration pathway, Daquinta et al. (1996) evaluated different concentrations of the growth regulator dicamba to induce the formation of embryogenic callus from leaf bases excised from *in vitro* grown plants of the cvs. Smooth Cayenne and Red Spanish. The nodular-type calli which developed were slow-growing and pale yellow in colour. Such calli produced somatic embryos, the latter developing into shoots on MS medium with  $2.5 \text{ mg l}^{-1}$  dicamba and  $0.5 \text{ mg l}^{-1}$  BAP. However, the frequency of plant regeneration was less than by organogenesis. Histological examination confirmed the embryogenic nature of the callus, while the structure of the somatic embryos was similar to that of their counterpart zygotic embryos. The authors did not discuss the further development of these somatic embryos, leaving it unclear as to whether such shoots developed into plants.

When leaves excised from axenic shoots of the Thai cv. Phuket were cultured on MS medium with  $3.0 \text{ mg l}^{-1}$  picloram, 58% of the leaf bases produced embryogenic callus within 35 days of culture (Sripaoraya et al. 2003), which was greater than the figure of 41% quoted earlier by Daquinta et al. (1996). Following subculture to the same medium, somatic embryos were initiated at the surface of the callus, about 21 days post-transfer. These somatic embryos developed into shoots when the tissues were placed on MS medium with  $1.0 \text{ mg l}^{-1}$  BA; 66% of individual embryogenic tissues each produced at least three shoots. Again, this frequency of shoot regeneration was considerably more than that (45%) reported previously for embryogenic tissues by Daquinta et al. (1996). Sripaoraya et al. (2003) estimated that about 6560 shoots could be regenerated from each explant per annum through somatic embryogenesis, with rooted plants being produced within 150 days of culture initiation.

Sripaoraya et al. (2003) also transferred embryogenic callus, induced on leaf bases cultured on MS medium with  $1.0 \text{ mg l}^{-1}$  picloram, or the same concen-

tration of 2,4-D, to liquid medium to initiate embryogenic cell suspensions. Subculture every 7 days maintained the cells in an embryogenic condition for at least 6 months. Cells harvested from picloram-supplemented liquid medium produced the most shoots (approx. 27 from 1.5 g fresh weight of cells) after transfer of tissues to MS medium with  $1.0 \text{ mg l}^{-1}$  BA and semi-solidified with 1.0% (w/v) SeaKem agarose. As in the case of shoots induced from leaf bases, those from embryogenic cell suspensions rooted on MS medium lacking growth regulators and were established *ex vitro*.

In an extension of their earlier investigations with bioreactors, Firoozabady and Moy (2004) developed friable embryogenic tissues, embryogenic cell clusters and chunky non-dispersable embryogenic tissues from leaf base and stem explants of *in vitro* grown shoots. These different tissues exhibited different growth rates. A comparison of shoot regeneration was made with crown tip meristems and leaf explants regenerating shoots by direct organogenesis. In some experiments, nodular globular structures were induced that also developed into shoots. Plants that originated by somatic embryogenesis exhibited 21% spininess when subsequently grown under field conditions compared to 5% for shoots regenerated by organogenesis. The friable embryogenic tissues and chunky non-dispersable embryogenic tissues were also used as targets for *Agrobacterium*-mediated transformation (Firoozabady et al. 2006).

#### 5.4 Somaclonal Variant Plants Regenerated from Cultured Tissues

Pineapple represents ideal material for mutation research as it sometimes exhibits spontaneous mutation (Leal and Coppens d'Eeckenbrugge 1996). For example, following mutagenesis, Wakasa (1979) observed extensive variation in leaf spination and pigmentation, wax deposition and foliage density in plants regenerated from callus. Variation in leaf spines and the dense foliage of some plants were attributed to the chimaeric nature of the donor plants. Most plants that differentiated from the syncarp were phenotypic variants. In contrast, plants regenerated from crowns or slips were predominantly normal in their morphology. Wakasa (1979) also related the variation in somatic mutation frequency to the type of callus initiated from the different explants. Axillary buds from crowns, suckers and slips produced globular structures that differentiated spontaneously into leaves, while syncarp-derived callus produced nodular, but undifferentiated structures, that persisted over many subcultures (Wakasa et al. 1978).

Commencing with a spiny chimaeric plant of the cv. Red Spanish, Liu et al. (1989) generated plants with fewer spines. Chimeras were frequent in this cv., with smooth sub-clones being obtained by traditional clonal selection. Since the segregation observed for spininess cannot be attributed to tissue culture *per se*, this phenotype may represent a case of clonal selection accelerated by *in vitro* multiplication. In more recent studies, Escalona et al. (1999) assessed the frequency of the spiny leaf form in vegetatively propagated plants of the cv.



Cayenne and in plants regenerated using their temporary immersion system described earlier. They reported about 6% phenotypic variation with respect to leaf spines, in contrast to the normal spiny-tipped leaf characteristic of this cv. In contrast, others have recorded a low frequency of variation in the cv. Cayenne, with only a limited number of variants being observed under field conditions in the French West Indies (Martinique) amongst a population of 40,000 in vitro-derived plants (Ventura et al. 1996). Interestingly, with the exception of three or four chlorophyll-deficient (albino) mutants, all the culture-derived plants reverted to a normal phenotype before flower induction. The difference in spininess of plants regenerated by organogenesis and somatic embryogenesis in the cv. Smooth Cayenne, reported by Firoozabady and Moy (2004), was probably attributed to somaclonal variation.

To date, agronomically useful variation has not been observed in plants regenerated from cultured tissues of Thai pineapple cvs., although it is possible that plants with variegated leaves could have potential value as ornamentals, provided leaf variegation is stable in micropropagated shoots. Plants regenerated from axillary or terminal buds of suckers showed the most somaclonal variation in terms of green/white striped leaves, with about 6–7% of micropropagated plants of the cvs. Phuket and Tradsithong exhibiting this characteristic. However, observations indicated that such leaf variegation was transitory in the cv. Phuket, with subsequent reversion to a green phenotype (Sripaoraya 2001).

## **6 Agronomic Improvement of Pineapple Using Genetic Manipulation: The Options**

### **6.1 Fusion of Isolated Protoplasts: Somatic Hybridisation and Cybridisation**

Conventional breeding, involving sexual hybridisation between species and cvs., sometimes aided by tissue culture based-technologies such as embryo rescue, is likely to generate elite pineapple germplasms. Similarly, the selection of naturally occurring or induced mutations, followed by clonal selection and the conventional multiplication and/or micropropagation of elite individuals, is almost certain to yield novel plants for commercial exploitation. In addition, in vitro-based approaches dependent upon reproducible cell-to-plant systems, can be exploited to mobilise genes into existing commercial cvs. of pineapple.

Somatic hybridisation involving the chemical and/or electrofusion of protoplasts isolated from different genera, species or cvs. enables plants to be generated with new combinations of nuclear and organellar genomes. Procedures have been described for the enzymatic isolation of viable protoplasts from a range of plants, followed by fusion of such isolated protoplasts and the subsequent selection of tissues from which hybrid plants have been regenerated (Davey et al. 2000b, c, 2005a, b, c). The generation of cytoplasmic hybrids



(cybrids), usually through a donor–recipient protoplast fusion approach, often involving irradiation to eliminate the nuclear genome of the donor partner, has also been discussed in these articles. Protoplast fusion permits the mobilisation of complex genetic traits without the need to isolate and to clone genetic material. Indeed, protoplast fusion technology forms a useful adjunct to conventional breeding approaches, since it generates unique combinations of nuclear and cytoplasmic genetic materials which are not possible by conventional sexual hybridisation.

Somatic hybridisation and cybridisation (Zubko et al. 2001) have been exploited to introgress traits of agronomic potential into plants, with examples being cited in several crops (Davey et al. 2005a, b, c). These techniques have considerable potential for the genetic improvement of pineapple. However, such approaches are not yet applicable to this crop since there are no reports, to date, of the development of reproducible protoplast-to-plant systems for *A. comosus*. Although protoplasts have been isolated from leaves of cultured shoots of *A. comosus* var. *comosus* (Sripaoraya 2001), it has been impossible to induce such protoplasts to develop into cells from which shoots can be regenerated through organogenesis or somatic embryogenesis, using the procedures established for tissues of explant origin. Considerably more effort needs to be directed towards the development of reliable and efficient protoplast-to-plant systems in pineapple as a prelude to gene transfer by somatic hybridisation and cybridisation. In this respect, approaches require evaluation, which, in turn, have been exploited to promote the growth and regeneration of protoplast-derived cells in other systems, such as exposure of protoplasts to high voltage electrical pulses following isolation and prior to their introduction into culture (Davey et al. 1996). Other parameters worthy of investigation include the culture of protoplasts in the presence of nurse cells (Horita et al. 2003), manipulation of the supply of respiratory gases through oxygen enrichment of the headspace, and the use of media supplements such as bovine haemoglobin and perfluorocarbon liquids (Lowe et al. 1998, 2003). The addition of surfactants to the culture medium (Lowe et al. 2001) needs to be evaluated. All of these approaches have been demonstrated to enhance significantly the growth and regeneration in culture of protoplasts of both dicotyledons and monocotyledons. Combinations of surfactants, haemoglobin and perfluorocarbon liquids are also worthy of investigation, since they often exert a synergistic effect in stimulating cell division and subsequent morphogenesis. Indeed, such innovative approaches could also be applied to stimulate shoot multiplication directly from explants of pineapple, as demonstrated in citrus (Cancino et al. 2001).

## 6.2 Transformation of Monocotyledons: Technology Transfer to Pineapple

During the last decade, considerable progress has been made in inserting and expressing genes in plants, with considerable emphasis on monocotyledons. In this respect, cereals have been the prime target for transgene insertion

because of their nutritional importance (Potrykus 2001), with three main transformation approaches being developed. Chronologically, these have involved the uptake of DNA into isolated protoplasts, Biolistic procedures and *Agrobacterium*-mediated gene transfer into totipotent cells and tissues. Other techniques and their utility have been discussed (Petolino 2002; Rakoczy-Trojanowska 2002). In all cases, gene delivery has been followed by the selection of transformed tissues and the regeneration of transgenic plants. Initially, the use of isolated protoplasts as recipients of foreign DNA was related to the availability of protoplast-to-plant systems for some monocotyledons, particularly rice (Davey et al. 2000a). Subsequently, Biolistic (particle bombardment) approaches were developed to circumvent labour-intensive protoplast systems, which were often difficult to establish and to repeat. More recently, *Agrobacterium*-mediated gene delivery has become the procedure of choice in many laboratories for the transformation of monocotyledons (Cheng et al. 2004), following the reports of Hiei et al. (1994, 1997), contrary to the earlier beliefs of many workers that monocotyledons were recalcitrant to *Agrobacterium*-mediated transformation. Undoubtedly, the success in transforming the major cereals by *Agrobacterium* has been related to improved technology in vector construction, particularly the development of superbinary vectors for efficient gene transfer to recipient plant cells (Barsby et al. 2001; Ingram et al. 2001; Ke et al. 2001; Rommens et al. 2004). There exists an extensive literature with many specific examples of the transformation of dicotyledons and monocotyledons, including representatives of fruit and grain crops, vegetables, medicinal plants, trees and ornamentals (Khachatourians et al. 2002). General introductions to this topic are presented in several excellent reviews (Zupan and Zambryski 1995; Tinland 1996; Christou 1997; Gheysen et al. 1998; Dunwell 2000; Newell 2000; Trieu et al. 2000; Dumas et al. 2001; Gelvin 2003; Valentine 2003) with a collection of edited protocols (Curtis 2004). These technologies are available to be exploited for pineapple.

### 6.3 *Agrobacterium*-Mediated Gene Transfer to Pineapple

As in the case of gene transfer by somatic hybridisation and cybridisation involving protoplast fusion, reports are limited relating to pineapple transformation. The absence of reproducible protoplast-to-plant systems in pineapple currently eliminates an approach involving the uptake of DNA into these naked cells. In the first report based on *Agrobacterium*-mediated transformation, Firoozabady et al. (1997) genetically manipulated the cv. Smooth Cayenne. Embryogenic tissues exhibiting different rates of growth and development were co-cultivated with the disarmed strain of *A. tumefaciens*, C58C1, harbouring a binary vector carrying either an *als* gene, conferring resistance to the selective herbicide chlorsulfuron (2-chloro-N[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino] benzenesulfonamide), or the neomycin phosphotransferase (*nptII*) gene. The latter conferred resistance in plant cells to the antibiotics neomycin,

kanamycin sulphate and geneticin (G418). The  $\beta$ -glucuronidase (*gus*) gene was also located on the T-DNA of the binary vector. Following co-cultivation with *Agrobacterium*, transformed tissues were selected on medium containing chlorsulfuron or G418. About 30 transformed callus lines were obtained per gram of fresh weight of embryogenic tissue inoculated with the bacterium. Transformed, embryogenic calli produced somatic embryos, which developed into transgenic plants. Transformation was confirmed by a GUS histochemical assay, molecular analysis using the polymerase chain reaction (PCR) and Southern hybridisation. A number of plants from several independently transformed lines were transferred to the glasshouse to evaluate their genetic stability.

Sripaoraya (2001) also attempted to transform Thai pineapple cvs. using *Agrobacterium* for gene delivery. *Agrobacterium tumefaciens* strain 1065 carrying a binary vector with the *gus* and *nptII* genes was used to inoculate embryogenic cell suspensions. This strain of *Agrobacterium* was chosen because of its supervirulent phenotype, the latter enabling the bacterium to transform a wide range of dicotyledons (Curtis et al. 1994). Although GUS-positive regions were observed on inoculated pineapple tissues, it was not possible to select transformed tissues on kanamycin-containing culture medium, possible because of the restricted extent of transformation, as indicated by limited histochemical (indigo) staining. More recently, success has been reported in transforming pineapple by *Agrobacterium*-mediated gene delivery (Firoozabady et al. 2006). Friable embryogenic tissues and chunky non-dispersable embryogenic tissues of the cv. Smooth Cayenne were co-cultivated with the disarmed strain C58 of *A. tumefaciens* carrying a binary vector with the *surB* gene for chlorsulfuron resistance or the *nptII* gene for resistance to geneticin (G418). These authors obtained 50–120 transgenic tissues from each gram of embryogenic cell clusters or embryogenic tissues, respectively, with transgenic tissues developing into plants by somatic embryogenesis. The efficiency of the transformation system enabled large populations of plants to be assessed for their clonal fidelity and somaclonal variation under glasshouse and field conditions. Clearly, this report represents a major advance in pineapple transformation technology.

#### **6.4 Particle Bombardment for Pineapple Transformation: Introduction of Herbicide Tolerance**

Several workers have evaluated particle bombardment to transform pineapple. In a preliminary report, Nan and Nagai (1998) employed leaf base-derived tissues (protocorm-like bodies) maintained in liquid medium as targets for gene delivery. Following bombardment with DNA constructs carrying *gus* and *nptII* genes, transformed tissues were selected by their ability to grow in liquid medium supplemented with G418, followed by plant regeneration on agar-solidified medium. An average of one transformation event was obtained

for one or two Petri dishes of bombarded tissues. Transgenic pineapple lines contained the *gus* and *nptII* genes inserted at one to two loci, as determined by Southern hybridisation. These transgenic lines were multiplied and plants transferred to the glasshouse for further evaluation. This procedure formed the basis for subsequent studies, by the same authors, of pineapple transformation, using agronomically important genes, such as those for disease resistance.

The *Agrobacterium* and microprojectile bombardment procedures reported by Firoozabady et al. (1997) and Nan and Nagai (1998) demonstrated transformation per se, but did not generate transgenic plants with potentially improved agronomic value. Likewise, Ko et al. (2000) described a transformation technique for the cv. Smooth Cayenne using leaf callus as a target for bombardment, with *gus* and *gfp* (green fluorescent protein) genes being exploited to optimise the conditions for transient and stable gene expression. G418-resistant transgenic callus and plants were obtained following co-transformation with constructs carrying the *nptII* gene. These authors recovered 15 independent transgenic GUS and GFP positive lines, with regenerated plants carrying multiple copies of the introduced genes. Ko et al. (2000) indicated that their transformation system was being exploited to introduce genes into pineapple to control post-harvest blackheart, based on inhibition of polyphenol oxidase expression. This preliminary report of Ko et al. (2000) indicated that subsequent field evaluations would determine the merit of Biolistic-mediated gene delivery for improving fruit quality of the ubiquitous 'standard' pineapple cv. Smooth Cayenne.

In other investigations, Sripaoraya et al. (2000, 2001b) introduced herbicide tolerance into the Thai pineapple cv. Phuket by microprojectile-mediated delivery of the plasmid AHC25 into the bases of leaves excised from micropropagated shoots. The plasmid AHC25 carried the *gus* and *bar* genes, both driven by the maize ubiquitin promoter. The *bar* gene from *Streptomyces hygroscopicus* codes for phosphinothricin acetyltransferase (PAT), which confers resistance to the non-selective herbicide bialaphos by acetylation of the free amide group of the active component, phosphinothricin (PPT). PAT also converts glufosinate ammonium, the active component of the commercially available herbicide Basta, to an inactive form. Transformed plants were recovered from bombarded leaf bases using a rapid shoot regeneration procedure developed by the same research group (Sripaoraya et al. 2003), eliminating the need for more complex regeneration pathways involving somatic embryogenesis. Thus, bombarded leaf bases were cultured on MS-based medium containing  $0.5 \text{ mg l}^{-1}$  2,4-D,  $2.0 \text{ mg l}^{-1}$  BA and  $0.5 \text{ mg l}^{-1}$  PPT, followed by transfer to MS medium with  $1.0 \text{ mg l}^{-1}$  BA and  $0.1 \text{ mg l}^{-1}$  PPT. Subsequently, regenerated plants were micropropagated on MS medium with  $1.0 \text{ mg l}^{-1}$  BA, but with the PPT concentration increased to  $2.0 \text{ mg l}^{-1}$ . Shoots were rooted on MS medium lacking growth regulators with the PPT concentration maintained at  $2.0 \text{ mg l}^{-1}$ . Micropropagated plants were transferred to the glasshouse. Integration and expression of the *bar* gene in the transgenic plants was confirmed by Southern analysis and reverse transcriptase (RT) PCR analyses, respectively.

Regenerated plants were assessed for their herbicide tolerance both in vitro and in the glasshouse. Studies in vitro involved the culture of plants on MS medium supplemented with Basta diluted to give 0, 3, 5, 7, 10, 15 or 20 mg l<sup>-1</sup> glufosinate ammonium. The herbicide tolerance of transgenic plants after 28 days of culture was compared with that of non-transformed plants. Concentrations in excess of 3.0 mg l<sup>-1</sup> of glufosinate ammonium in the culture medium resulted in an inhibition of growth and loss of pigmentation in non-transformed plants within 4 days of exposure to the herbicide. In contrast, all PPT-tolerant plants remained green and developed normally in the presence of the herbicide. Transgenic and non-transformed plants, which had been acclimatised to glasshouse conditions for 75 days, were sprayed to run-off with aqueous solutions of Basta diluted to contain 100, 200, 400, 600, 800, 1200 or 1,400 mg l<sup>-1</sup> of glufosinate ammonium; plant responses were scored 14 days after spraying. Application of the herbicide to the leaves of non-transformed plants resulted in necrosis after 4 days, followed by browning within a further 14 days of growth and subsequent death of the plants. However, leaves of PPT-tolerant plants remained green and the plants continued to grow vigorously, even when treated with herbicide solution containing 1,400 mg l<sup>-1</sup> of glufosinate ammonium.

In Thailand, the broad-spectrum herbicide Basta is used to control native weeds in pineapple plantations, typically at a concentration of 1,200 mg l<sup>-1</sup> glufosinate ammonium. Interestingly, the transgenic plants generated by Sripaoraya et al. (2001b) survived spraying with herbicide at concentrations in excess of those normally applied in the field. Thus, such transgenic plants could be exploited in agricultural systems employing chemical weed control. As Sripaoraya et al. (2001b) emphasised, the generation of large populations of transgenic plants from a limited number of selected individuals presents few problems, since a single transgenic pineapple plant can be micropropagated without difficulty to generate in excess of  $5.0 \times 10^4$  clonal individuals within 12 months. An advantage of introducing such genetically manipulated pineapple plants into commercial cultivation is that it could encourage more producers to utilise Basta as a herbicide. The latter is less costly, less toxic and more biodegradable than bromacil, which is used currently by the majority of farmers. Improved weed control in pineapple plantations should result in reduced plant competition, increased fruit yields and, indirectly, a reduction in insect pests such as mealy bugs and ants.

Molecular analyses confirmed the presence of up to eight *bar* gene integration sites in PPT-tolerant plants of the cv. Phuket. In plants that rely upon seed production for multiplication, multiple transgene inserts may be problematical and the inheritance of transgenes difficult to predict. However, this should not present a problem in pineapple, which is propagated vegetatively, provided plants that exhibit high transgene expression are selected for multiplication. Three hundred and twenty transgenic, herbicide-tolerant plants of the cv. Phuket and the same number of non-transformed plants were evaluated under experimental field conditions in Thailand, for transgene stability, gene expres-

sion, fruit characteristics and yield (Sripaoraya et al. 2006). Seven months after transfer to the field, plants remained tolerant to 1,600 ml rai<sup>-1</sup> of Basta (1 rai = 0.16 ha = 1,600 m<sup>2</sup>), equivalent to a concentration of 4 g l<sup>-1</sup> glufosinate ammonium. Molecular analyses involving PCR amplified a 460 bp fragment of the *bar* gene from the DNA of transgenic plants after the latter had been in the field for 210 days, with Southern analysis confirming the stable integration of the transgene in such plants. RT-PCR confirmed expression of the *bar* gene in transgenic plants after 380 days in the field. The time to flowering and fruit harvest was the same for both transgenic and non-transformed plants, since both were sprayed at the same time with the ethylene-generating compound Authel (ethephon; 2:chloroethelphosphonic acid) at 225 and 230 days following transfer to the field. Importantly, fruit yield was not affected by transgene insertion and expression; transgenic plants produced crisp, aromatic fruits comparable to those of their non-transformed counterparts. It should be possible to extend Biolistic technology to other commercially important Thai cvs.

### 6.5 Genetic Manipulation of Pineapple for Disease Resistance

The introduction of disease resistance into pineapple is a realistic target for the immediate future, particularly if the physiological and molecular basis of plant-pathogen interactions are well understood (Honée 1999). To date, various strategies have been devised to engineer disease-resistant crops, including expression of plant disease-response pathogen components, genes encoding plant, fungal and bacterial hydrolytic enzymes and those encoding defence-response elicitors (Pink and Puddephat 1999; Stuiver and Custers 2001). Examples of the introduction of fungal and bacterial resistances into crops are provided in reports by Lee et al. (2002) and Yuan et al. (2002); these authors also give extensive literature citations.

As already indicated, viral-induced wilt disease constrains pineapple production, making the generation of virus-resistant plants an important target for genetic manipulation technologies in the future. A recent example of the benefits of genetically engineered virus resistance is provided by studies with papaya (*Carica papaya*), a plant cultivated extensively in the tropics and subtropics for its fruit (Chen et al. 2001). Following cloning of the papaya ringspot virus (PRSV) replicase gene, these workers introduced the gene into the papaya cv. Tai-nong-2 by *A. tumefaciens*-mediated transformation of embryogenic calli, generating virus-resistant plants and seed progeny. Thus, a similar viral gene cloning approach could be adopted for pineapple wilt disease, followed by transformation of commercial cvs. of pineapple by particle bombardment or, possibly, in the longer term, by *Agrobacterium*-mediated gene delivery. Since insects such as mealy bugs transmit viral-induced wilt disease in pineapple, the generation of insect-resistant plants through expression of genes encoding proteinase inhibitors (Xie et al. 2002) or lectins (Wu et al. 2002) offers another approach to reduce, albeit indirectly, the incidence of this disease.



Botella and Fairbairn (2005) reported that nematode resistance has been introduced into plants based on an anti-feeding defence strategy. Cysteine proteinase inhibitors are small proteins that can pass through the stylet and into the gut of plant parasitic nematodes, where the inhibitors interfere with nutrient uptake, inhibiting the growth of the nematode(s) in transgenic plants. Information relating to this technology and its possible application in pineapple is available at the appropriate website (<http://www.biology.leeds.ac.uk/centres/liba/cps>) and in a publication that details a multi-institution, inter-disciplinary, international programme to genetically engineer pineapple (Rohrbach et al. 2000). Importantly, the programme was driven by participation of all partners, including Hawaii's pineapple industry. A large-scale parasitic nematode gene sequencing project was also initiated at Washington University ([www.nematode.net](http://www.nematode.net)), which includes over 18,000 sequences of the pineapple root-knot nematode pathogen.

In terms of virus resistance, the genome of pineapple mealybug wilt-associated closterovirus-2 (PMWaV-2) was cloned from double-stranded RNA isolated from diseased pineapple, and the nucleotide sequence of the virus determined. Genome organisation of the virus was typical of the monopartite closteroviruses; phylogenetic analysis revealed that mealybug-transmitted PMWaV-2 was closely related to other mealybug-transmitted viruses of the family *Closteroviridae*, PMWaV-2 showing the greatest sequence homology to grapevine leafroll-associated virus-3 (Melzer et al. 2001). Such information will be crucial in designing virus-resistant plants through genetic manipulation approaches.

Blackheart disease arises when fruits are exposed to low temperatures in the field or when refrigerated following harvesting. The centre of the fruit becomes dark brown in colour because of the action of polyphenol oxidase (PPO) on accumulated phenolic compounds, the latter being induced by chilling injury. Thus, inactivation of PPO genes could reduce or eliminate discoloration of the fruit (Graham et al. 2000). In order to test this hypothesis, the latter authors generated transgenic plants by particle bombardment of totipotent callus initiated from leaf bases of in vitro grown plants and by *Agrobacterium*-mediated gene delivery to leaf bases. Botella and Fairbairn (2005) noted, in their review of the potential of biotechnological approaches to pineapple improvement, that PPO expression was down-regulated in transgenic pineapple plants under field conditions. In studies that followed those of Graham et al. (2000), Ko et al. (2006) introduced the *gus* and *gfp* reporter genes into totipotent leaf callus by particle bombardment in order to establish the transformation procedure. Subsequently, the *ppo* gene from pineapple, together with constructs carrying the *nptII* gene conferring geneticin (G418) resistance, were introduced into target tissues. The authors regenerated 15 independent *gus* and *gfp* positive lines from 8 experiments and 22 *ppo* positive lines from 11 experiments, with Southern blot analysis confirming the presence of transgenes in regenerated plants. At the time of submission of the report for publication, the *ppo* and *gus*



positive plants were being field trialled for gene expression, genetic integrity and organoleptic characteristics (Ko et al. 2006).

Although expression of the *bar* gene for herbicide tolerance did not have any detrimental influence on the performance of pineapple under field conditions (Sripaoraya et al. 2006), other workers have reported biochemical side effects of genetic transformation during the period of in vitro plantlet hardening. Thus, Yabor et al. (2006) compared non-transformed plantlets with those transformed with the *bar*, chitinase and *ap 24* genes. The chitinase gene from *Phaseolus vulgaris* and the *ap 24* gene from *Nicotiana tabacum* are antifungal genes, these being introduced into pineapple in an attempt to reduce crop losses incited by *Phytophthora nicotianae* var. *parasitica*. Both transgenic and non-transformed plants were similar with respect to height, weight and peroxidase activity, but significant changes were recorded in aldehydes, including malondialdehyde, chlorophylls, free and cell wall-linked phenolics and proteins. It remains to be seen whether similar changes are also expressed following transfer of plants to glasshouse and field conditions.

## 6.6 Genetic Manipulation of Pineapple to Control Flowering

As already discussed, plantation-grown pineapples are normally sprayed with ethylene-generating chemicals to induce uniformity in flowering. The latter is essential to facilitate fruit harvesting and to minimise associated costs. Nevertheless, some plants still flower naturally, with fruit maturing prior to normal harvest. In efforts to develop strategies to control flowering, Botella et al. (2000) cloned and characterised ACC synthase (*acacc-1* and *acacc-2*) and ACC oxidase (*acaco-1*) genes. These workers also demonstrated that these genes were involved in ethylene biosynthesis, induced during fruit ripening and, consequently, associated with early flowering. They generated transgenic plants with sense and antisense copies of the *acacc-2* gene in experiments to control early natural flowering. Transgenic plants were evaluated in the first trial to employ this patented ACC technology for pineapple, with encouraging results in relation to the flowering of transgenic plants compared to controls under field conditions (Botella and Fairbairn 2005). ACC synthase was also targeted in the research programme summarised by Rohrbach et al. (2000).

## 7 Conclusions

Undoubtedly, pineapple will remain a fruit of major economic importance. Although considerable effort has been directed toward genetic improvement of the crop through conventional hybridisation at the cv. and inter-specific cross levels, it is clear that global production still relies heavily upon a relatively limited number of cvs. which have been selected for their agronomic characters. Interestingly, the genetic composition of some of these cvs. and species is

unclear, although biochemical and molecular markers have provided useful information in relation to the parentage of a range of pineapple germplasms (Duval et al. 2001; Sripaoraya et al. 2001a).

There still remains a need to generate new cvs. and to introgress genes from a diverse range of germplasms into the cultivated crop. In this respect, somatic hybridisation and cybridisation have much to offer, provided robust protoplast-to-plant systems can be developed for pineapple. The simple technique of embryo rescue should also be exploited more extensively to recover potentially interesting sexual hybrids. In vitro approaches have facilitated the multiplication of elite individuals, shoot regeneration occurring by either organogenesis or somatic embryogenesis. Most regenerated plants are true-to-type, although a limited number may exhibit somaclonal variation, some forms of which may be useful to the breeder and would not be deemed the product of genetic manipulation technology. This may facilitate rapid producer/consumer acceptance. Mutagenesis also has a somewhat similar role in the generation of novel germplasm.

Genetic transformation provides a means of introducing specific genes, such as those for herbicide, insect and disease tolerances, into well-characterised cvs. The success of this approach is reflected in the introduction of herbicide tolerance into the Thai pineapple cv. Phuket, with gene expression making such plants strong contenders for evaluation under field conditions. Other traits that could be introduced include increased shelf-life for harvested fruit, possibly mediated by expression of genes that are activated as fruit senescence proceeds through modification of the concentrations of endogenous growth regulators (McCabe et al. 2001). The long-term expression of transgenes in vegetatively propagated pineapple plants should present less difficulties compared to transgene expression in seed-propagated crop species.

Whilst biotechnological approaches have considerable potential for the agronomic improvement of pineapple, the long-term advantages of procedures such as transformation will be governed, to some extent, by consumer acceptability of genetically manipulated fruit. Barsby et al. (2001) and Ingram et al. (2001) have discussed these difficulties in relation to cereals, such as transgenic wheat, and have provided a critique of some of the potential risks perceived by the public in relation to transgenic crops. Other authors have discussed the relationship of genetically manipulated crops to food safety, environmental issues and human health (Azevedo and Araujo 2003; Haselberger 2003; König et al. 2004; Goldstein et al. 2005). Likewise, Falk et al. (2002) presents a detailed overview of the public perception of transgenic crops and emphasises that there is a requirement to increase consumer understanding of food production and processing through the distribution of accurate, factual information on biotechnology. Similarly, the provision of adequate information should be extended to farmers and workers in the food industry who have direct contact with consumers. Linked to consumer acceptance of transgenic crops are, undoubtedly, issues of intellectual property protection (Means 2002) and political and economic consequences (Phillips and Khachatourians 2002).

Such difficulties also apply to pineapple, with the need to generate consumer confidence for genetically manipulated fruit. Indeed, the acceptability of genetically manipulated pineapple will be essential in the long term, especially in view of the exploitation of transgenic plants as 'bioreactors' for the synthesis of pharmaceutically important products (Fischer and Emans 2000). Such products, if synthesised in pineapple, for example, could be administered orally by consumption of the unprocessed fruit, as suggested for other fruits and vegetables (Fooks 2000; Giddings et al. 2000; Richter et al. 2001; Daniell et al. 2002; Raskin et al. 2002; Schillberg et al. 2003).

## References

- Aghion D, Beauchesne G (1960) Utilisation de la technique de culture sterile d'organes pour obtenir des clones d'*Ananas*. *Fruits* 15:464–466
- Almeida WAB de, de Matos AP, da Souza AS (1996) Effects of benylaminopurine (BAP) on in vitro proliferation of pineapple (*Ananas comosus* (L.) Merr.). *Acta Hort* 425:235–239
- Aradhya MK, Zee F, Manshardt RM (1994) Isozyme variation in cultivated and wild pineapple. *Euphytica* 79:87–99
- Azevedo JL, Araujo WL (2003) Genetically modified crops, environmental and human health concerns. *Mutation Res* 544:223–233
- Barboza SBSC, Graciano-Ribeiro D, Teixeira JB, Portes TA, Souza LAC (2006) Leaf anatomy of micropropagated pineapple plants. *Pesq Agropec Brasileira* 41:185–194
- Barsby T, Power JB, Freeman J, Ingram HM, Livesey NL, Risacher T, Davey MR (2001) Transformation of wheat. In: Bonjean AP, Angus WJ (eds) *The world wheat book. A history of wheat breeding*. Lavoisier, Paris, pp 1081–1103
- Bartholomew DP, Paull RE, Rohrbach KG (2003) *The pineapple. Botany, production and uses*. CABI, Wallingford, Oxon
- Be LV, Debergh PC (2006) Potential low-cost micropropagation of pineapple (*Ananas comosus*). *South Afr J Bot* 72:191–194
- Benega R, Isidrón M, Arias E, Cisneros A, Martínez J, Torres I, Hidalgo M, Borroto CG (1996a) In vitro germination and callus formation in pineapple hybrid seeds (*Ananas comosus* (L.) Merr.). *Acta Hort* 425:243–246
- Benega R, Isidrón M, Arias E, Cisneros A, Martínez J, Companioni L, Borroto CG (1996b) Plant regeneration from pineapple ovules (*Ananas comosus* (L.) Merr.). *Acta Hort* 425:247–250
- Botella JR, Fairbairn DJ (2005) Present and future potential of pineapple biotechnology. *Acta Hort* 622:23–28
- Botella JR, Cavallaro AS, Cazzonelli CI (2000) Towards the production of transgenic pineapple to control flowering and ripening. *Acta Hort* 529:115–122
- Cabral JRS, Cunha GAP, Rodrigues M (1984) Pineapple micropropagation. *Anais do VII Congresso Brasileiro de Fruticultura* 1:124–127
- Cancino GO, Gill MIS, Anthony P, Davey MR, Power JB, Lowe KC (2001) Pluronic F-68 enhanced shoot regeneration in a potentially novel *Citrus* rootstock. *Art Cells Blood Subs Immob Biotechnol* 29:317–324
- Carneiro LA, Araújo RFG, Brito GJM, Fonseca MHPB, Costa A, Crocomo OJ, Mansur E (1999) In vitro regeneration from leaf explants of *Neoregelia cruenta* (R. Graham) L.B. Smith, an endemic bromeliad from eastern Brazil. *Plant Cell Tissue Organ Cult* 55:79–83
- Cazzonelli CI, Cavallaro AS, Botella JR (1998) Cloning and characterisation of ripening-induced ethylene biosynthetic genes from non-climacteric pineapple (*Ananas comosus*) fruits. *Aust J Plant Physiol* 25:513–518

- Chan YK, Coppens d'Eeckenbrugge G, Sanewski GM (2003) Breeding and variety development. In: Bartholomew DP, Paull RE, Rohrbach KG (eds) *The pineapple: botany, production and uses*. CABI, Wallingford, Oxon, pp 33–55
- Chen G, Ye CM, Huang JC, Yu M, Li BJ (2001) Cloning of the papaya ringspot virus (PRSV) replicase gene and generation of PRSV-resistant papayas through the introduction of the PRSV replicase gene. *Plant Cell Rep* 20:272–277
- Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev Biol – Plant* 40:31–45
- Christou P (1997) Rice transformation: bombardment. *Plant Mol Biol* 35:197–203
- Chu CC (1978) The N6 medium and its application to another culture of cereal crops. *Proc Symp Plant Tissue Culture*, Science Press, Beijing, pp 43–50
- Collins JL (1960) *The pineapple. Botany, cultivation, and utilization*. Leonard Hill, London
- Coppens d'Eeckenbrugge G, Leal F (2003) Morphology, anatomy and taxonomy. In: Bartholomew DP, Paull RE, Rohrbach KG (eds) *The pineapple: botany, production and uses*. CABI, Wallingford, Oxon, pp 13–32
- Cote F, Domergue R, Folliot M, Bouffin J, Marie F (1991) Micropropagation in vitro de l'ananas. *Fruits* 46:359–366
- Curtis IS (2004) *Transgenic crops of the world. Essential protocols*. Kluwer, Dordrecht
- Curtis IS, Power JB, Blackhall NW, de Laat AMM, Davey MR (1994) Genotype-independent transformation of lettuce using *Agrobacterium tumefaciens*. *J Exp Bot* 45:1441–1449
- Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci* 7:84–91
- Daquinta MA, Cisneros A, Rodríguez Y, Escalona M, Pérez M, Luna I, Borroto CG (1996) Embriogénesis somática en piña (*Ananas comosus* (L.) Merr.). *Acta Hort* 425:243–246
- Davey MR, Blackhall NW, Lowe KC, Power JB (1996) Stimulation of plant cell division and organogenesis by short-term, high-voltage electrical pulses. In: Lynch PT, Davey MR (eds) *Electrical manipulation of cells*. Chapman and Hall, New York, pp 273–286
- Davey MR, Ingram H, Azhakanandam K, Power JB (2000a) The genetic transformation of rice and maize. In: Morris PC, Bryce JH (eds) *Cereal biotechnology*. Woodhead, Cambridge, pp 43–69
- Davey MR, Power JB, Lowe KC (2000b) Plant protoplasts. In: Spier RA (ed) *Encyclopedia of cell technology*. John Wiley, New York, pp 1034–1043
- Davey MR, Lowe KC, Power JB (2000c) Protoplast fusion for the generation of unique plants. In: Spier RA (ed) *Encyclopedia of cell technology*. John Wiley, New York, pp 1090–1096
- Davey MR, Anthony P, Power JB, Lowe KC (2005a) Plant protoplasts: status and biotechnological perspectives. *Biotechnol Adv* 23:131–171
- Davey MR, Anthony P, Power JB, Lowe KC (2005b) Plant protoplast technology: status and applications. *SIVB Congr Symp Proc on Thinking outside the cell. In Vitro Cell Dev Biol-Plant*. 41:202–212
- Davey MR, Anthony P, Power JB, Lowe KC (2005c) Plant protoplast technology: current status. *Acta Physiol Plant* 27:117–129
- de Wald MG, Moor GA, Sherman WB (1988a) Identification of pineapple cultivars by isozyme genotypes. *J Am Soc Hortic Sci* 113:935–938
- de Wald MG, Moor GA, Sherman WB (1988b) Production of pineapple plants in vitro. *Plant Cell Rep* 7:535–537
- de Wald MG, Moor GA, Sherman WB (1992) Isozymes in *Ananas* (pineapple): genetics and usefulness in taxonomy. *J Am Soc Hortic Sci* 117:491–496
- Dumas F, Duckely M, Pelczar P, Van Gelder P, Hohn B (2001) An *Agrobacterium* VirE2 channel for transferred-DNA transport into plant cells. *Proc Natl Acad Sci USA* 98:485–490
- Dunwell JM (2000) Transgenic approaches to crop improvement. *J Exp Bot* 51:487–496
- Duval MF, Coppens d'Eeckenbrugge G, Ferreira FR, Cabral JRS, Bianchetti L de B (1997) First results from joint EMBRAPA-CIRAD *Ananas* germplasm collecting in Brazil and French Guiana. *Acta Hort* 425:137–144

- Duval MF, Noyer JL, Perrier X, Coppens d'Eeckenbrugge G, Hamon P (2001) Molecular diversity in pineapple assessed by RFLP markers. *Theor Appl Genet* 102:83–90
- Escalona M, Lorenzo JC, Espinosa P, González B, Luna I, Fundora Z, Cintras M, Hernández Z (1995) Effect of paclobutrazol on *Ananas comosus* (L.) Merr. shooting. I. Paclobutrazol concentration. *Adv Modern Biotechnol* 3:2–4
- Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y, Borroto CG (1999) Pineapple (*Ananas comosus* L. Merr.) micropropagation in temporary immersion systems. *Plant Cell Rep* 18:743–748
- Falk MC, Chassy BM, Harlander SK, Hoban TJ, McGloughlin MN, Akhlaghi AR (2002) Food biotechnology: benefits and concerns. *J Nutrit* 132:1384–1390
- Firoozabady E, Gutterson N (2003) Cost-effective in vitro propagation methods for pineapple. *Plant Cell Rep* 21:844–850
- Firoozabady E, Moy Y (2004) Regeneration of pineapple plants via somatic embryogenesis and organogenesis. In *Vitro Cell Dev Biol-Plant* 40:67–74
- Firoozabady E, Heckert M, Oeller P, Gutterson N (1997) Transformation and regeneration of transgenic pineapple plants. *Proc 5th Int Congr Plant Mol Biol*, Singapore, Abstract No. 1358
- Firoozabady E, Heckert M, Gutterson N (2006) Transformation and regeneration of pineapple. *Plant Cell Tissue Organ Cult* 84:1–16
- Fischer R, Emans N (2000) Molecular farming of pharmaceutical products. *Transgenic Res* 9:279–299
- Fitchet M (1990) Clonal propagation of Queen and Smooth Cayenne pineapple. *Acta Hort* 275:261–266
- Fitchet-Purnell M (1993) Maximum utilisation of pineapple crowns for micropropagation. *Acta Hort* 334:325–330
- Fooks AR (2000) Development of oral vaccines for human use. *Curr Opin Mol Ther* 2:80–86
- Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50:151–158
- Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Mol Biol Rev* 67:16–37
- Gheysen G, Angenon G, van Montagu M (1998) *Agrobacterium*-mediated plant transformation: a scientifically intriguing story with significant applications. In: Lindsay K (ed) *Transgenic plant research*. Harwood, Amsterdam, pp 1–33
- Giddings G, Allison G, Brooks D, Carter A (2000) Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol* 18:1151–1155
- Goldstein DA, Tinland B, Gilbertson LA, Staub JM, Bannon GA, Goodman RE, McCoy RL, Silanovich A (2005) Human safety and genetically modified plants: a review of antibiotic resistance markers and future transformation selection strategies. *J Appl Microbiol* 99:7–23
- Gonzalez-Olmedo JL, Fundora Z, Molina LA, Abdunnour J, Desjardins Y, Escalona M (2005) New contributions to propagation of pineapple (*Ananas comosus* L. Merr) in temporary immersion bioreactors. In *Vitro Cell Dev Biol-Plant* 41:87–90
- Graham M, Ko L, Hardy V, Robinson S, Sawyer B, O'Hare T, Jobin M, Dahler J, Underhill S, Smith M (2000) The development of blackheart resistant pineapples through genetic engineering. *Acta Hort* 529:133–136
- Hamasaki RM, Purgatto E, Mercier H (2005) Glutamine enhances competence for organogenesis in pineapple leaves cultivated in vitro. *Braz J Plant Physiol* 17:383–389
- Haselberger AG (2003) GM food: the risk assessment of immune hypersensitivity reactions covers more than allergenicity. *Food Agric Environ* 1:42–45
- Hepton A, Hodgson AS (2003) Processing. In: Bartholomew DP, Paull RE, Rohrbach KG (eds) *The pineapple. Botany, production and uses*. CABI, Wallingford, Oxon, pp 281–290
- Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271–282
- Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol Biol* 35:205–219

- Hirimburegama K, Wijesinghe LPJ (1992) In vitro growth of *Ananas comosus* L. Merr. (pineapple) shoot apices on different media. *Acta Hort* 319:203–208
- Honée G (1999) Engineering resistance against fungal pathogens. *Eur J Plant Pathol* 105:319–326
- Horita M, Morohashi H, Komai F (2003) Production of fertile somatic hybrid plants between Oriental hybrid lily and *Lilium × formolongi*. *Planta* 217:597–601
- Ingram HM, Livesey NL, Power JB, Davey MR (2001) Genetic transformation of wheat: progress during the 1990s into the millennium. *Acta Physiol Plant* 23:221–239
- Jones JB, Murashige T (1974) Tissue culture propagation of *Aechmea fasciata* Baker and other Bromeliads. *Comb Proc Int Plant Prop Soc* 24:117–126
- Ke J, Khan R, Johnson T, Somers DA, Das A (2001) High-efficiency gene transfer to recalcitrant plants by *Agrobacterium tumefaciens*. *Plant Cell Rep* 20:150–156
- Khachatourians GG, McHughen A, Scorza R, Nip W-K, Hui YH (2002) Transgenic plants and crops. Marcel Dekker, New York
- Khatun MM, Khanam D, Hoque MA, Quasem A (1997) Clonal propagation of pineapple through tissue culture. *Plant Tissue Cult* 7:143–148
- Kiss E, Kiss J, Gyulai G, Heszky LE (1995) A novel method for rapid micropropagation of pineapple. *HortScience* 30:127–129
- Ko HL, Graham MW, Hardy VG, Jobin M, O'Hare TJ, Smith MK (2000) Transformation of pineapple using biolistics. *Proc 6th Int Congr Plant Mol Biol*, Québec, Abstract No. S03-64
- Ko HL, Campbell PR, Jobin-Décor MP, Eccleston KL, Graham MW, Smith MK (2006) The introduction of transgenes to control blackheart in pineapple (*Ananas comosus* L.) cv. Smooth Cayenne by microprojectile bombardment. *Euphytica* 150:387–395
- König A, Cockburn A, Crevel RWR, Debruyne E, Graftstroem R, Hammerling U, Kimber I, Knudson I, Kuiper HA, Peijnenburg AACCM, Penninks AH, Poulsen M, Schauzu M, Wal JM (2004) Assessment of the safety of foods derived from genetically modified crops. *Food Chem Toxicol* 42:1047–1088
- Lakshmi Sita G, Singh R, Layer CPA (1974) Plantlets through shoot tip culture in pineapple. *Curr Sci* 43:724
- Leal F, Coppens d'Eeckenbrugge G (1996) Pineapple. In: Janick J, Moore JN (eds) *Fruit breeding, vol I. Tree and tropical fruits*. John Wiley, New York, pp 515–557
- Lee YH, Yoon IS, Suh SC, Kim HI (2002) Enhanced disease resistance in transgenic cabbage and tobacco expressing a glucose oxidase gene from *Aspergillus niger*. *Plant Cell Rep* 20:857–963
- Liu LJ, Rosa-Marquez E, Lizareli E (1989) Smooth leaf (spineless) Red Spanish pineapple (*Ananas comosus*) propagated in vitro. *J Agric Univ Puerto Rico* 73:301–311
- Lowe KC, Davey MR, Power JB (1998) Perfluorochemicals: their applications and benefits to cell culture. *TIBTECH* 16:272–277
- Lowe KC, Anthony P, Davey MR, Power JB (2001) Beneficial effects of Pluronic F-68 and artificial oxygen carriers on the post-thaw recovery of cryopreserved plant cells. *Artif Cells Blood Substit Immobil Biotechnol* 29:297–316
- Lowe KC, Anthony P, Power JB, Davey MR (2003) Novel approaches for regulating gas supply to plant systems in vitro: applications and benefits of artificial gas carriers. *In Vitro Cell Dev Biol-Plant* 39:557–566
- Mapes MO (1973) Tissue culture of Bromeliads. *Comb Proc Int Plant Prop Soc* 23:47–55
- Mathews VH, Rangan TS (1979) Multiple plantlets in lateral buds and leaf explants of in vitro cultures of pineapple. *Sci Hort* 11:319–328
- Mathews VH, Rangan TS (1981) Growth and regeneration of plantlets in callus cultures of pineapple. *Sci Hort* 14:227–234
- Mathews VH, Rangan TS, Narayanaswamy S (1976) Micro-propagation of *Ananas sativus* in vitro. *Z Pflanzenphysiol* 79:450–454
- McCabe MS, Garratt LC, Schepers F, Jordi WJRM, Stoopen GM, Davelaar E, van Rhijn JHA, Power JB, Davey MR (2001) Effects of P<sub>sag12</sub>-IPT gene expression on development and senescence in transgenic lettuce. *Plant Physiol* 127:505–516



- McKeon TA, Fernandez-Maculet JC, Yang SF (1995) Biosynthesis and metabolism of ethylene. In: Davies P (ed) Plant hormones – physiology, biochemistry and molecular biology, 2nd edn. Kluwer, Dordrecht, pp 118–139
- Means KA (2002) Industrial perspectives. In: Khachatourians GG, McHughen A, Scorza R, Nip W-K, Hui YH (eds) Transgenic plants and crops. Marcel Dekker, New York, pp 305–309
- Melzer MJ, Karasev AV, Sether EM, Hu JS (2001) Nucleotide sequence, genome organization and phylogenetic analysis of pineapple mealybug wilt-associated virus-2. J Gen Virol 82:1–7
- Moore GA, de Wald MG, Evans MH (1992) Micropropagation of pineapple (*Ananas comosus* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 18: high-tech and micropropagation. Springer, Berlin Heidelberg New York, pp 460–469
- Moyle R, Fairbairn DJ, Ripi J, Crowe M, Botella JR (2005) Developing pineapple fruit has a small transcriptome dominated by metallothionein. J Exp Bot 56:101–112
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
- Murashige T, Tucker DPH (1969) Growth factor requirements of *Citrus* tissue culture. Proc 1st Int Citrus Symp 3:1155–1161
- Nan G-L, Nagai C (1998) Genetic transformation of pineapple (*Ananas comosus* [L.] Merr.) via particle bombardment. In Vitro Cell Dev Biol 34:55-A, Abstract No. 1052
- Newell CA (2000) Plant transformation technology. Developments and applications. Mol Biotechnol 16:53–65
- Noyer JL (1991) Intérêt des RFLP pour l'amélioration des variétés cultivées du genre *Ananas*. PhD Thesis, Université Montpellier 2 Sciences et Techniques du Languedoc, Montpellier
- Noyer JL, Lanaud C, Duval MF, Coppens d'Eeckenbrugge G (1996) RFLP study on rDNA variability in the *Ananas* genus. Acta Hort 425:153–160
- Oeller PW, Gutterson N (1997) The ACC synthase gene family in pineapple. Proc 5th Int Congr Plant Mol Biol, Singapore, Abstract No. 1357
- Petolino JF (2002) Direct DNA delivery into intact cells and tissues. In: Khachatourians GG, McHughen A, Scorza R, Nip W-K, Hui YH (eds) Transgenic plants and crops. Marcel Dekker, New York, pp 137–143
- Phillips PWB, Khachatourians GG (2002) Political and economic consequences. In: Khachatourians GG, McHughen A, Scorza R, Nip W-K, Hui YH (eds) Transgenic plants and crops. Marcel Dekker, New York, pp 311–325
- Pink D, Puddephat I (1999) Deployment of disease resistance genes by plant transformation – a “mix and match” approach. Trends Plant Sci 4:71–75
- Potrykus I (2001) The “golden rice” tale. In Vitro Cell Dev Biol-Plant 37:93–100
- Py C, Lacoëuilhe JJ, Teisson C (1987) Pineapple, cultivation and uses. Maisonneuve and Larose, Paris
- Rahman KW, Amin MN, Azad MAK (2001) In vitro rapid clonal propagation of pineapple, *Ananas comosus* (L.) Merr. Plant Tissue Cult 11:47–53
- Rakoczy-Trojanowska M (2002) Alternative methods of plant transformation – a short review. Cell Mol Biol Lett 7:849–858
- Rangan TS (1984) Pineapple. Handbook of plant cell culture. Crop Species 3:373–382
- Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B (2002) Plants and human health in the twenty-first century. Trends Biotechnol 20:522–531
- Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci USA 98:11539–11554
- Rohrbach KG, Christopher D, Hu J, Paull R, Sipes B, Nagai C, Moore P, McPherson M, Atkinson H, Levesley A, Oda C, Fleisch H, McLean M (2000) Management of a multiple goal pineapple genetic engineering program. Acta Hort 529:111–113
- Rohrbach KG, Leal F, Coppens d'Eeckenbrugge G (2003) History, distribution and world production. In: Bartholomew DP, Paull RE, Rohrbach KG (eds) The pineapple. Botany, production and uses. CABI, Wallingford, Oxon, pp 1–12



- Rommens CM, Humara JM, Ye J, Yan H, Richael C, Zhang L, Perry R, Swords K (2004) Crop improvement through modification of the plant's own genome. *Plant Physiol* 135:421–431
- Rosa-Marquez E, Lizardi E (1987) Callus induction and regeneration from Red Spanish pineapple. *Phytopathology* 77:1711
- Ruas PM, Ruas CF, Fairbanks DJ, Andersen WR, Cabral JRS (1995) Genetic relationships among four varieties of pineapple, *Ananas comosus*, revealed by random amplified polymorphic DNA (RAPD) analysis. *Brazilian J Genet* 18:413–416
- Schillberg S, Fischer R, Emans N (2003) Molecular farming of recombinant antibodies in plants. *Cell Mol Life Sci* 60:433–445
- Smith LB, Downes RJ (1979) *Bromelioidees (Bromeliaceae)*. *Flora Neotropica*, Monograph 14, Pt. 3
- Srinivasa Rao NK, Dore Swamy R, Chacko EK (1981) Differentiation of plantlets in hybrid embryo callus of pineapple. *Sci Hort* 15:235–238
- Sripaoraya S (2001) Genetic manipulation of pineapple (*Ananas comosus*). PhD Thesis, University of Nottingham
- Sripaoraya S, Marchant R, Ingram HM, Davey MR, Lowe KC, Power JB (2000) Transformation of pineapple (*Ananas comosus*) via particle bombardment. *J Exp Bot* 51 (Suppl): Abstract P9.29, 55
- Sripaoraya S, Blackhall NW, Marchant R, Power JB, Lowe KC, Davey MR (2001a) Relationships in pineapple by random amplified polymorphic DNA (RAPD) analysis. *Plant Breed* 120:265–267
- Sripaoraya S, Marchant R, Power JB, Davey MR (2001b) Herbicide-tolerant pineapple (*Ananas comosus*) produced by microprojectile bombardment. *Ann Bot* 88:597–603
- Sripaoraya S, Marchant R, Power JB, Davey MR (2003) Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L.). *In Vitro Cell Dev Biol-Plant* 39:450–454
- Sripaoraya S, Keawsompong S, Insupa P, Power JB, Davey MR, Srinives P (2006) Genetically manipulated pineapple: transgene stability, gene expression and herbicide tolerance under field conditions. *Plant Breed* 125: 411–413
- Stuiver MH, Custers JHHV (2001) Engineering disease resistance in plants. *Nature* 411:865–868
- Teng WL (1997) An alternative propagation method of *Ananas* through nodule culture. *Plant Cell Rep* 16:454–457
- Teixeira SL, Ribeiro JM, Teixeira MT (2006) Influence of NaClO on nutrient medium sterilization and on pineapple (*Ananas comosus* cv. Smooth Cayenne) behaviour. *Plant Cell Tissue Organ Cult* 86:375–378
- Thomson KG, Thomas JE, Dietzgen RG (1998) Retrosposon-like sequences integrated into the genome of pineapple, *Ananas comosus*. *Plant Mol Biol* 38:461–465
- Tinland B (1996) The integration of T-DNA into plant genomes. *Trends Plant Sci* 1:178–183
- Trietue AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA, Shin HS, Chiou TJ, Katagi H, Dewbre GR, Weigel D, Harrison MJ (2000) Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. *Plant J* 22:531–541
- Valentine L (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. *Plant Physiol* 133:948–955
- Ventura JA, Zambolim L, Chaves GM (1996) Tissue culture technique for rapid clonal propagation of pineapple cultivars. *Acta Hort* 425:161–166
- Vinterhalter B, Vinterhalter D (1994) True-to-type in vitro propagation of *Aechmea fasciata* Baker. *Sci Hort* 57:253–263
- Wakasa K (1979) Variation in the plants differentiated from the tissue culture of pineapple. *Jpn J Breed* 29:13–22
- Wakasa K (1989) Pineapple. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol. 5: trees. Springer, Berlin Heidelberg New York, pp 13–29
- Wakasa K, Koge Y, Kudo M (1978) Differentiation from in vitro cultures of *Ananas comosus*. *Jpn J Breed* 28:113–121

- White PR (1963) The cultivation of animal and plant cells, 2nd edn. Ronald Press, New York
- Wu A, Sun X, Pang Y, Tang K (2002) Homozygous transgenic rice lines expressing GNA with enhanced resistance to the rice sap-sucking pest *Laodelphax striatellus*. *Plant Breed* 121:93–95
- Xie XZ, Huang MJ, Wu NH (2002) Transgenic tobacco plants harbouring tomato proteinase inhibitor II gene and their insect resistance. *Prog Natl Sci* 12:198–203
- Yabor L, Arzola M, Aragón, Hernández M, Arencibia A, Lorenzo JC (2006) Biochemical side effects of genetic transformation of pineapple. *Plant Cell Tissue Organ Cult* 86:63–67
- Yuan H, Ming X, Wang L, Hu P, An C, Chen Z (2002) Expression of a gene encoding trichosanthin in transgenic rice plants enhances resistance to fungus blast disease. *Plant Cell Rep* 20:992–998
- Zepeda C, Sagawa Y (1981) In vitro propagation of pineapple. *HortScience* 16:495
- Zubko MK, Zubko EL, Ruban AV, Adler K, Mock HP, Misera S, Gleba YY, Grimm B (2001) Extensive developmental and metabolic alterations in cybrids *Nicotiana tabacum* (plus *Hyoscyamus niger*) are caused by complex nucleo-cytoplasmic incompatibility. *Plant J* 25:627–639
- Zupan JR, Zambryski P (1995) Update on plant transformation. Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol* 107:1041–1047

## I.6 Watermelon

P. ELLUL<sup>1</sup>, C. LELIVELT<sup>2</sup>, M.M. NAVAL<sup>3</sup>, F.J. NOGUERA<sup>1</sup>, S. SANCHEZ<sup>1</sup>,  
A. ATARÉS<sup>4</sup>, V. MORENO<sup>4</sup>, P. CORELLA<sup>1</sup>, and R. DIRKS<sup>2</sup>

### 1 Introduction

The genus *Citrullus* Schrad. includes the commercial crop species *Citrullus lanatus* [(Thunb.) Matsum. and Nakai], which is commonly known as watermelon. Watermelon is an economically important vegetable crop of tropical and temperate parts of the world (Table 1). Asia produces 87% of the world watermelon crop (91,790,226 mt), with the most important contribution from China (66,434,289 mt), followed by Turkey (4,250,000 mt) and Iran (1,902,000 mt). In the European Union, watermelon is cultivated in countries bordering the Mediterranean, where Spain (714,000 mt), Greece (6,50,000 mt) and Italy (528,084 mt) are the main producers (FAOSTAT 2003).

Watermelon is appreciated by consumers for its texture, sweetness and flavour of the flesh. It is a good source of vitamins (C and A) and nutrients such as potassium, iron and calcium. Watermelon also contains a high amount of lycopene, a carotenoid molecule which, over recent years, has gained considerable interest in diets with respect to its potential as an antioxidant. In watermelon flesh, the amount of lycopene ranges from 23.0 to 72.0 mg g<sup>-1</sup> wet weight, where, for example, in fresh tomato, the values are between 8.8 and 42.0 mg g<sup>-1</sup> wet weight (Fraser and Bramley 2004).

### 2 Watermelon Origin and Diversity

The genus *Citrullus* of the Cucurbitaceae family consists of four species native to the Old World (Robinson and Decker-Walters 1997) and three that are indigenous to Namibia (Arnold and De Wet 1993). *Citrullus ecirrhosus* Cogn. is a perennial species endemic to the coastal Namibian Desert (Meeuse 1962), while *Citrullus rehmii* De Winter, a recently described annual species (De Winter 1990), has a limited distribution confined to the western escarpment.

<sup>1</sup> Rijk Zwaan Ibérica, Paraje El Mamí, Carretera De Viator S/N, 04120 La Cañada (Almería), Spain, e-mail: p.ellul@rijkwzaan.es

<sup>2</sup> Rijk Zwaan Breeding BV, 1e Kruisweg 9, 4793 RS Fijnaart, The Netherlands

<sup>3</sup> IVIA (Instituto Valenciano De Investigaciones Agrarias), Carretera Moncada – Náquera, Km. 4,5 Apartado Oficial, 46113 Moncada (Valencia), Spain

<sup>4</sup> IBMCP (Instituto De Biología Molecular Y Celular De Plantas), Avenida De Los Naranjos S/N, 46022 Valencia, Spain

**Table 1.** Watermelon production (FAO 2003, <http://faostat.fao.org/default.aspx>)

	Area harvested (ha)	Average yield (kg ha <sup>-1</sup> )	Production (mt)
World	3,469,871	26,454	91,790,226
Asia	2,678,618	29,766	79,731,111
Europe	351,844	11,302	3,976,469
Africa	171,072	20,726	3,545,659
South America	136,286	9,670	1,317,935
Central America and Caribbean	65,861	20,977	1,381,587
North America	60,860	28,722	1,748,000
Oceania	5,330	16,785	89,465

*Citrullus colocynthis* is a perennial herb common in the central desert of the United Arab Emirates (Wester 1989) and also grows widely in the Arabian and Sahara Deserts and in Sudan (Al-Ghaithi et al. 2004). In several countries of West Africa, especially in Nigeria, *C. colocynthis* is a wild watermelon plant, cultivated as a crop interplanted with maize, cassava and yam (Lagemann et al. 1975).

Various scenarios have been proposed for the origin of the domesticated watermelon, *C. lanatus*, from its wild progenitor (Bates and Robinson 1995; Robinson and Decker-Walters 1997). The African origin of *C. lanatus* is almost certain (Pitrat et al. 1999) because of the diversity of spontaneous forms of *C. lanatus* and the presence of wild relative species on this continent. Mallick and Masui (1986) and Esquinas-Alcazar and Gulick (1983) have proposed Central Africa and the Kalahari Desert as the centre of origin of cultivated watermelon. Moreover, watermelon has a long history of cultivation in Africa and the Middle East and has been planted in the Nile Valley since the second millennium BC (Zohary and Hopf 1988). Watermelon may have been domesticated in Egypt and western Asia around 2000 BC and was known very early on in Central Asia (Pitrat et al. 1999). *Citrullus* spp. cultivation may have evolved independently in different regions (Maggs-Kölling et al. 2000) and Hindustan (an area encompassing India, Nepal, Burma, Thailand and Pakistan) and has also been proposed as a centre of domestication of the crop (Zeven and de Wet 1982).

By the 10th century AD the crop was grown in China and southern Russia. Watermelon was then introduced to the New World by the Spaniards in the 16th century and rapidly became popular with Native Americans (Robinson and Decker-Walters 1997). The plant was first cultivated in hot and dry countries (tropical and Mediterranean), but is also cultivated under hot and humid climatic conditions (Pitrat et al. 1999).

### 3 Watermelon Cultivars and Germplasm

Depending on the type of fertilization, watermelon varieties fall into three broad classes: (1) open-pollinated varieties, (2)  $F_1$  hybrids and (3) triploid or seedless watermelons. Open-pollinated varieties are developed through several generations of selection, generally based upon yield, quality characteristics and disease resistance. The open-pollinated varieties have true-to-type seed and are less expensive than  $F_1$  hybrid varieties.

$F_1$  hybrids are developed from two inbred lines that have been selfed for several generations and then crossed, with the subsequent seed sold to growers.  $F_1$  hybrids generally exhibit increased uniformity of type, homogeneous time of harvest and an increased yield (20–40 %) when compared with open-pollinated varieties. For the grower, the disadvantages of  $F_1$  hybrid seed are the cost and the availability, considering that  $F_1$  hybrid varieties will generally change from year to year. Most open-pollinated varieties and  $F_1$  hybrids are diploid ( $2n = 2x = 22$ ).

The third type of cultivar is the triploid ( $2n = 3x = 33$ ) or seedless watermelon. Although triploid watermelons are referred to as seedless, they are not truly seedless, but rather have undeveloped seeds that are soft and edible. These rudimentary small seeds are consumed along with the flesh, just as immature seeds are eaten in cucumber. The triploid or seedless watermelons were first obtained and described in Japan by crossing female tetraploid and male diploid plants (Kihara 1951). The resulting triploid plants are sterile and do not produce viable seed. Triploids are developed by creating first tetraploid plants by doubling the chromosome number and crossing them with diploid watermelon (Andrus et al. 1971). Tetraploid seeds can be germinated immediately upon harvest and their ploidy confirmed using different methods cited in this chapter. The seed stock of confirmed tetraploids is expanded by repeated self-pollination. Because tetraploids exhibit reduced fertility (Lower and Johnson 1969) (first generation tetraploids may produce only 5–20 seeds per fruit) and limited fruit production per plant (maximum of 3–4 fruits per plant with seeds), as many as 10 years may be required to produce the quantity of seed needed to satisfy the commercial demand for new triploid seed (Compton et al. 2004). In addition, the production of triploid watermelons is more expensive than that of  $F_1$  hybrids (as much as 5–10 times) because of the high cost of the seed, the establishment of the crop from transplants and the necessary presence of a pollinator variety planted with the triploid variety (flowers of triploid plants lack sufficient viable pollen to induce normal fruit set). An adequate bee population is also essential to ensure sufficient pollen transfer. The resulting triploid plants are sterile and do not produce viable seed.

The modern watermelon cultivars could also be grouped according to fruit characteristics, such as shape (from round to oblong), rind color or pattern (light to dark green with or without stripes), fruit size and flesh colors (dark red, red or yellow). For example, oblong fruits with dark stripes on a light background in the 10–16 kilo range are called Jubilee types after the popular

Jubilee variety. Watermelons of similar shape and size as Jubilee but with a light green rind are called Charleston Gray types for similar reasons. Watermelons in the range of 8–13 kilos with a striped rind are Crimson Sweet types. Watermelons known as Sugar Baby types produce round fruits of 6–7 kilos, with a dark green rind and deep red flesh.

## 4 Breeding Watermelon for Chromosome Doubling

Considering that triploid seedless fruits are currently preferred by most consumers globally (Marr and Gast 1991; Ellul 2002), the first interest of chromosome doubling in watermelon is related to the production of tetraploid lines. Currently, only a few tetraploid breeding lines are used to produce the number of triploid hybrids available to producers. Considerable effort needs to be devoted to the development of an efficient method and resolving the difficulties associated with the production of tetraploid breeding lines, such as cytogenetic instability amongst generations and ploidy chimeras. This currently limits the genetic diversity amongst tetraploid parents for triploid hybrid production, eventually resulting in a narrow germplasm, which may pose a crop production risk for growers.

Chromosome doubling in watermelon is also related to the production of doubled haploids (i.e., homozygous lines). In fact, an important restriction to the use of haploid plants in breeding programmes is the low rate of spontaneous chromosome duplication observed during the andro- or gynogenetic regeneration process. Antimitotic agents, such as colchicine, are widely used *in vitro* to induce chromosome duplication (Herman 1997). For watermelon, immersion of *in vitro*-grown plants in colchicine is most commonly used (Sari et al. 1999a). In this technique, haploid plants or their single node cuttings are immersed in colchicine solution, washed with water and transplanted into growth media.

Independent of the breeding requirement, the most important aspect consists of evaluating accurately the ploidy level of the regenerated plants before and after the duplication treatment. To distinguish watermelon plants on the basis of their ploidy, indirect techniques (macro- and microscopic traits or flow cytometry analysis) have been proposed as an alternative to the classical method of chromosome counting.

### 4.1 Determination of the Ploidy of Watermelon Plants

#### 4.1.1 *Direct Method by Chromosome Counting*

For several decades, it has been known that in determining chromosome number or ploidy, the classic method of counting chromosomes has been the most accurate. In watermelon, several procedures for screening chromosome

number have been described (Skorupska and Allgood 1990; Sari et al. 1999b). However, this cytogenetic approach is time-consuming and requires a well-equipped laboratory and a qualified work team. In addition, the reliability of the root tip chromosome counting is relative, considering the high level of endopolyploidy and mixoploidy observed in root tips (see Sect. 4.2.3). For example, in roots of haploid watermelon plants, Sari et al. (1999b) described the occurrence of a few diploid cells (22 chromosomes) amongst predominantly haploid cells (11 chromosomes) in the same root tip.

4.1.2 Indirect Methods Based on Macro- and Microscopic Traits

When cytogenetic methods are ineffective or unavailable, several indirect methods could be used to evaluate the ploidy level in plants. Morphological traits, such as flower size (tetraploids generally produced larger flowers; see Fig. 1 and Table 2) or seed size and shape (tetraploid seeds are generally larger and thicker), may also be used in order to identify polyploids. A microscopic method, such as the counting of the numbers of chloroplasts per guard cell in leaf epidermis previously stained with silver nitrate (Ellul et al. 1999) or fluorescein diacetate (Compton et al. 1999), is also a valuable alternative for ploidy determination. This method has been used in watermelon grown in vitro, under glasshouse conditions or in the field (McCuistion and Elmstrom 1993; Compton et al. 1999, 2004; Ellul et al. 1999, 2000; Sari et al. 1999b; Ellul 2002). For example, our analysis performed with Sugar Baby type cultivars showed that diploid and tetraploid plants possess 11–13 and 19–23 chloroplasts per guard cell pair, respectively (Fig. 2). Sari et al. (1999a) determined the ploidy of haploid and diploid watermelon plants of the cvs. Sugar Baby and Halep Karasi, and observed that the number of chloroplasts of the guard cells in the haploids and diploids were 6–7 and 11–12, respectively.

**Table 2.** Diameter (in mm) of reproductive organs in male and female flowers of diploid and tetraploid watermelon plants grown under glasshouse conditions and obtained from seeds or regenerated by in vitro culture (somaclones and primary transformants)

Origin of plants (ploidy level)	Female flowers		Male flowers	
	Corolla	Ovary	Corolla	Stamen
<i>From seeds</i>	33.1 ± 0.39 <sup>a</sup>	8.9 ± 0.0 <sup>a</sup>	38.2 ± 0.31 <sup>a</sup>	6.5 ± 0.06 <sup>a</sup>
Diploid plants				
<i>In vitro culture</i>	34.0 ± 0.38 <sup>a</sup>	9.5 ± 0.07 <sup>a</sup>	38.3 ± 0.32 <sup>a</sup>	6.4 ± 0.06 <sup>a</sup>
Diploid somaclones				
Diploid transformants	34.5 ± 0.35 <sup>a</sup>	9.5 ± 0.07 <sup>a</sup>	38.3 ± 0.31 <sup>a</sup>	6.5 ± 0.06 <sup>a</sup>
Tetraploid somaclones	45.6 ± 0.35 <sup>b</sup>	13.6 ± 0.11 <sup>b</sup>	52.1 ± 0.30 <sup>b</sup>	8.4 ± 0.10 <sup>b</sup>
Tetraploid transformants	45.7 ± 0.33 <sup>b</sup>	14.2 ± 0.10 <sup>b</sup>	51.9 ± 0.31 <sup>b</sup>	8.6 ± 0.09 <sup>b</sup>

<sup>a,b</sup>Different letters indicate statistically significant differences with *p* > 95%





Fig. 1. Morphological characteristics of male (m) and female (f) flowers from tetraploid ( $2n = 4x = 44$ ) and diploid ( $2n = 2x = 22$ ) plants; transversal section of the carpels (c)

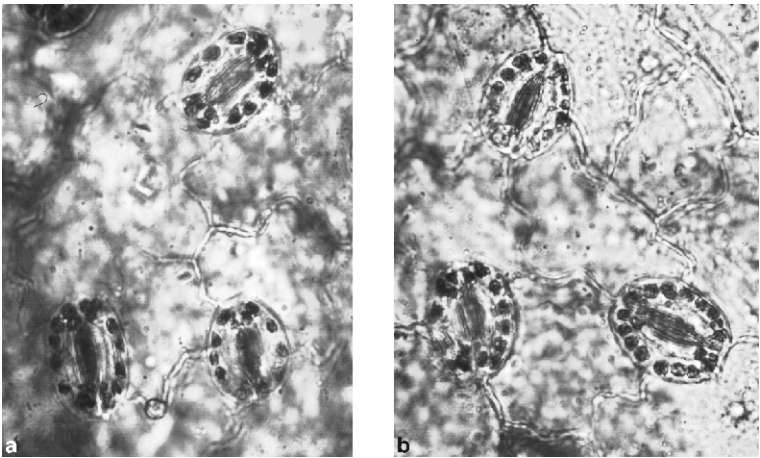


Fig. 2. Stained chloroplasts ( $\text{AgNO}_3$  10%) in guard cell pairs of the abaxial epidermis from leaves of diploid (a) and tetraploid (b) watermelon plants ( $\times 1250$ )

#### 4.1.3 Indirect Method Based on Amount of DNA and Flow Cytometry

Considering that conventional chromosome counting is time-consuming and that morphological trait analysis is a rapid screening method, but varies with genotype (Gray and Elmstrom 1991; McCuiston and Elmstrom 1993; Compton et al. 1996), flow cytometry offers a simple and accurate alternative. This technique involves the analysis of fluorescence and light-scattering properties of single particles (i.e., nuclei) during their passage within a narrow, precisely defined, liquid stream (Galbraith et al. 1983; Dolezel 1991). Heller (1973) was the first to use flow cytometry for DNA analysis in plant cells, and the method was subsequently used in other plant species (Dolezel et al. 1989; Galbraith 1990; Arumuganathan and Earle 1991). In watermelon, several collections of haploid, diploid, triploid and tetraploid plants have been identified by flow cytometric analysis (Zhang et al. 1994; Ellul et al. 1999, 2000, 2003a; Sari et al. 1999b).

However, in spite of the fact that the equipment is expensive and in limited supply, flow cytometry offers several advantages over traditional counting techniques. Flow cytometry is a powerful and reliable technique for estimating plant nuclear DNA content, since it permits sensitive measurements of fluorescence intensity of large numbers of stained nuclei within seconds. For example, in a routinely ploidy level analysis, approximately 30 samples of leaves could be checked in less than 1 h. A ploidy analyzer by flow cytometry is simple to use, less time-consuming than chromosome counting or other indirect methods, and permits the screening of any plant tissue. Altogether, these aspects explain why flow cytometry is being used increasingly for large-scale ploidy screening (Dolezel and Bartos 2005).

### 4.2 Duplication of the Chromosome Number in Watermelon

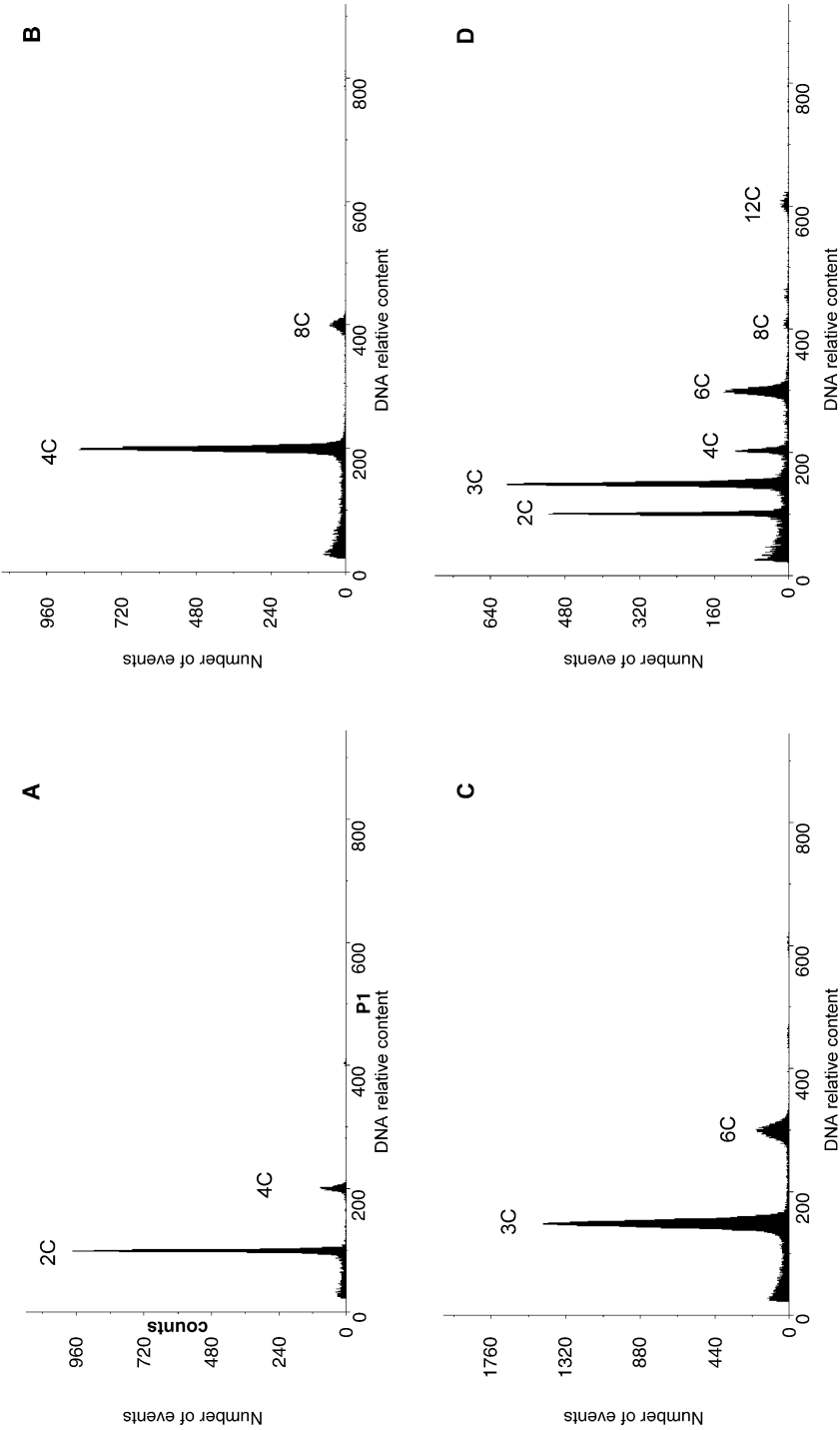
#### 4.2.1 Antimitotic Treatments

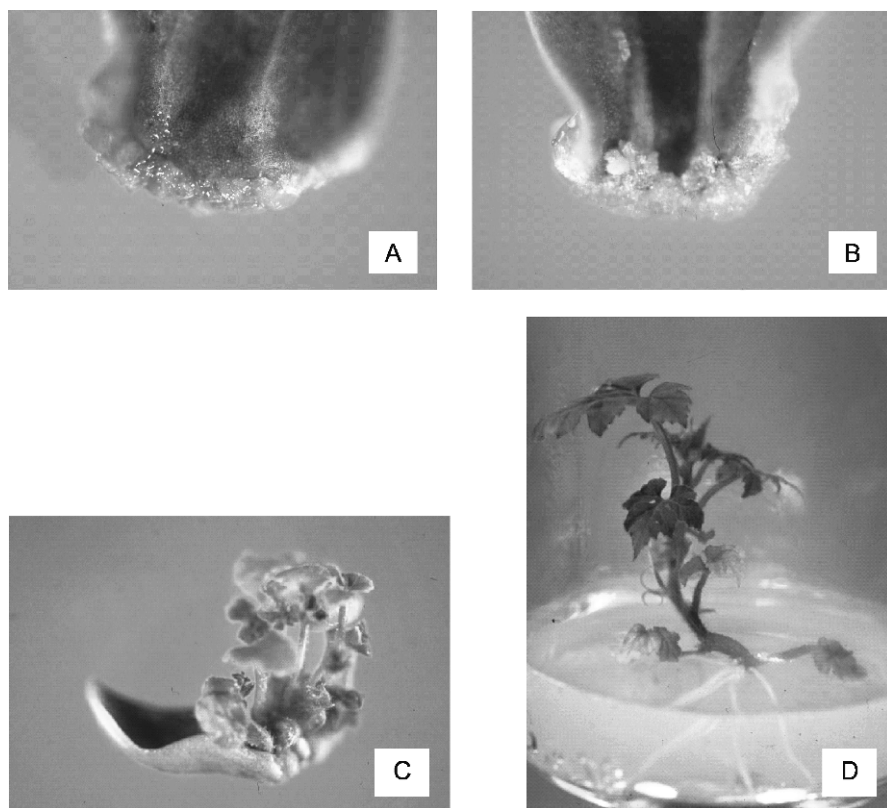
Tetraploid watermelon plants are obtained by treating young emerged diploid seedlings with colchicine, an antimitotic alkaloid which allows the duplication of the chromosome number (Andrus et al. 1971). Colchicine is an allelopathic compound produced by *Colchicum autumnale*, well known for inhibiting the formation of spindle fibers, and effectively arresting mitosis at the metaphase stage (Herman 1997). Since chromosomes have already multiplied but cell division is arrested, polyploid cells are created. However, colchicine is carcinogenic and generally less effective than herbicides with a similar mode of action, such as the dinitroanilines oryzalin and trifluralin (Morejohn et al. 1987; Van Tuyl et al. 1992; Zhao and Simmonds 1995; Hansen and Andersen 1996; Kato 1999; Petersen et al. 2002; Takamura et al. 2002; Chauvin et al. 2003; Kermani et al. 2003).

#### 4.2.2 Improving Somaclonal Variation

It is well established that genetic changes of various kinds occur amongst plants regenerated from tissue culture (i.e., somaclones). This variation, designated as somaclonal variation, ranges from point mutation to chromosomal arrangements, aneuploidy and polyploidy (Larkin and Scowcroft 1981). The production of polyploid regenerants from tissue culture has been reported for many plant species (Veilleux and Johnson 1998) and has potential application to establish a large number of new watermelon tetraploid breeding lines. As described in other species, polyploidy amongst watermelon regenerated plants obtained by adventitious shoot regeneration is common (Zhang et al. 1994; Compton et al. 1996; Ellul et al. 2000, 2003a). Depending on the regeneration procedure and the genotypes selected, Compton et al. (1996) reported that 5–20% of shoots regenerated from cotyledon explants of five different diploid cultivars could be tetraploid. These results are in agreement with the polyploidization rates we obtained with Sugar Baby and Crimson Sweet types by applying a three-step organogenic regeneration protocol (Fig. 3). Unlike treatment of meristems with colchicine, a high proportion (90%) of regenerants from tissue culture are non-chimeric, true-breeding tetraploids (Compton et al. 1996; Jaworski and Compton 1997; Ellul 2002). Tetraploid regenerants from tissue culture display reduced fertility compared to diploids of the same genotype, but are more fertile than tetraploids obtained from colchicine treatment (Compton et al. 2004). Putative tetraploids from tissue culture can be screened using the methods stated above (see Sect. 4.1). However, in order to avoid loss of time and space in the glasshouse, flow cytometry can be applied to leaves proceeding from regenerated plants grown in vitro (Fig. 4). An excellent report has been published by Compton et al. (2004) giving regeneration protocol details, and both adventitious shoot organogenesis and somatic embryogenesis could be used for tetraploid plant regeneration. However, plants regenerated by somatic embryogenesis are advantageous because they generally possess improved shoot and root vascular connections and somatic embryogenic systems usually display a higher embryo and plant regeneration rate (Ammirato 1987). The advantage of in vitro techniques is that tissue culture can be used to produce non-chimeric true breeding tetraploids efficiently from a wide range of diploid cultivars.

► **Fig. 3.** Histograms of number of nuclei per channel as a function of relative fluorescence intensity resulting from flow cytometry analysis of nuclei stained with DAPI. Nuclei were isolated from leaf samples of diploid (A), tetraploid (B) and triploid (C) plants. Mixed population of nuclei (D) isolated from cotyledons of diploid and triploid plantlets

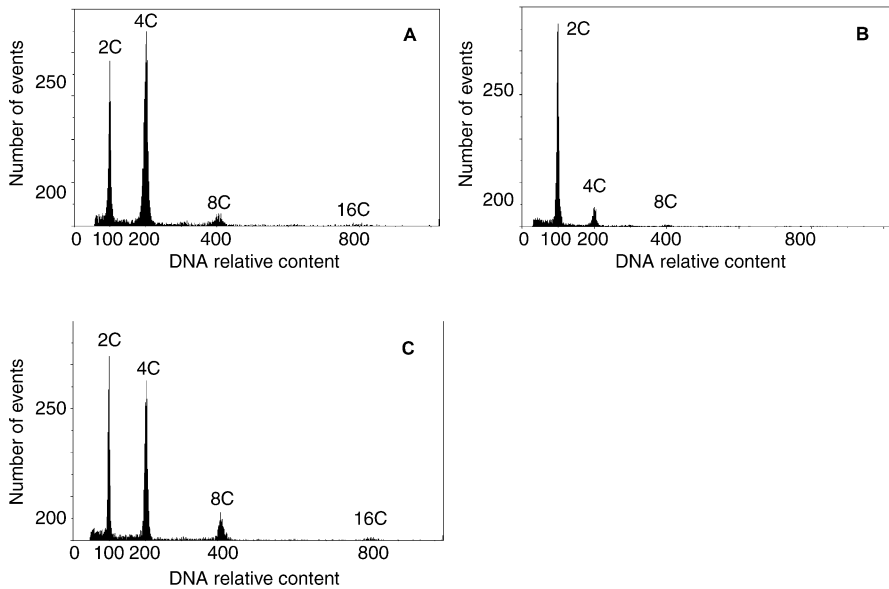




**Fig. 4.** Shoot organogenesis in watermelon. **A** Organogenic callus at the cut edge of the proximal end of a cotyledon explant. **B** Adventitious shoot buds formed 10 days after culture initiation. **C** Adventitious shoot formed 30 days after culture initiation. **D** Rooted individual regenerated plant 6 weeks after culture initiation

#### *4.2.3 Improving Polysomy During Seedling Development*

The phenomenon of mixoploidy, i.e., the presence in somatic tissue of different ploidy levels, has been known for a long time in animals and plants (D'Amato 1984). At the cellular level, DNA endoreduplication is a common feature of eukaryotes and leads to endopolyploid cells by repeated cycles of DNA synthesis without intervening cell division (Nagl 1976). Apart from its basic interest, the phenomenon of mixoploidy/polyploidy is of great importance for *in vitro* applications. Considering that mixoploidy develops in a regulated fashion, the study of the evolution of the pattern of ploidy in certain organs during natural seedling development may provide information concerning the genetic stability of the tissue frequently used as explant source (i.e., cotyledon, leaf, hypocotyl). Knowledge of the degree of mixoploidy in the explant tissue source could be valuable for the regeneration of polyploid plants in view of the inten-



**Fig. 5.** Different polysomatic patterns in cotyledon (A), leaf (B) and hypocotyl (C) isolated from watermelon plantlets

sive use in recent years of molecular and cellular methods for plant breeding and varietal improvement in watermelon.

The watermelon flow cytometry analysis performed in our laboratory revealed the presence of cells with DNA contents ranging from 2–64C in varying proportions (1C is the DNA content after meiosis in a haploid cell) depending on the individual organ (Fig. 5) and the age of the donor plant. These results showed the occurrence of polyploidization and the development of mixoploidy in most of the somatic tissue which was used for tissue culture, such as cotyledon, leaf or hypocotyl (Fig. 5).

However, the seedless watermelons are generally obtained by crossing tetraploid and diploid plants; they have also been produced using plant growth regulators, but this method has occasionally resulted in deformed fruits and poses food safety problems. The induction of reciprocal translocations of chromosomes is another approach with the same objective, but it was discarded because the development of chromosome translocation lines and commercial cultivars based on these lines is difficult, and fruits are not completely seedless. Another method for producing seedless watermelon has been described by Sugiyama and Morishita (2000), using soft-X-irradiated pollen (doses ranging from 400–1,000 Gy), which does not affect fruit weight, shape, rind thickness or days to maturation when compared to controls.

## 5 Genetic Engineering and Watermelon Breeding Purposes

From a breeding point of view, genetic engineering can be a useful tool, but it is absolutely necessary to achieve expression of the candidate gene(s) in the crop species, breeding lines and cultivars where the agronomic trait is to be evaluated. For watermelon, several transformation protocols have been described for different cultivars in order to initiate a breeding programme by using a genetic engineering approach.

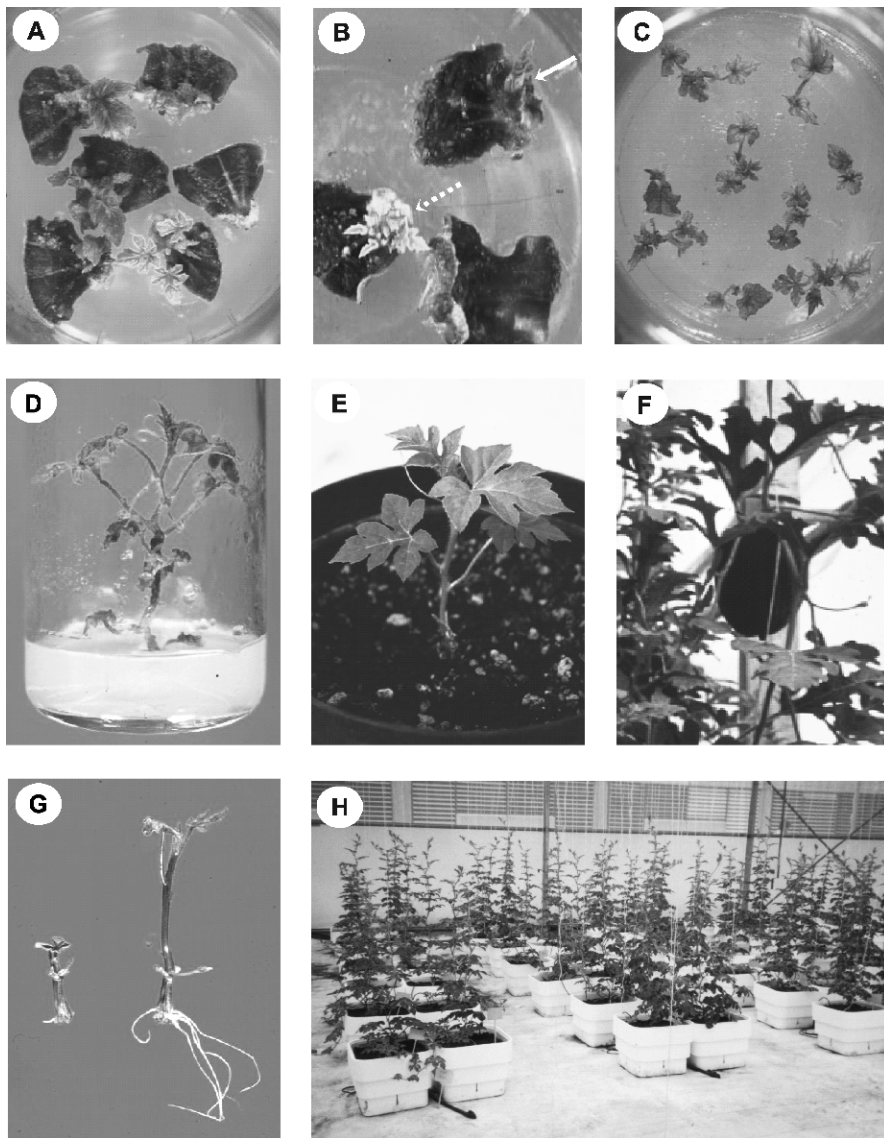
### 5.1 Transformation Procedures and Selection of Transformants

Stable genetic transformation has been reported for the first time in *C. lanatus* using cotyledon explants and the disarmed strain LBA4404 of *Agrobacterium tumefaciens* (Choi et al. 1994). This strain contained the binary vector pBI121 carrying the *nos* promoter-neomycin phosphotransferase (*nptII*) chimeric gene for selection and the cauliflower mosaic virus (CaMV) 35S promoter- $\beta$ -glucuronidase (GUS) gene (*gusA*) as a reporter gene. In further transformation experiments, the recovery of watermelon transgenic shoots has generally been achieved using the *nptII* gene for selection in callus induction medium containing kanamycin (Tricoli et al. 2002; Ellul et al. 2003a). Additionally, the phosphomannose isomerase positive selection method has been successful with watermelon with regard to producing transgenic plants (Reed et al. 2001).

In our watermelon transformation experiments (Fig. 6), cotyledon explants inoculated with *A. tumefaciens* LBA4404 carrying pBI121 and selected with 125 or 175 mg l<sup>-1</sup> kanamycin showed transformation efficiencies of 5.3 and 3.6%, respectively, for the cv. Dulce Maravilla, and 3.1 and 2.6%, respectively, for cv. Crimson Sweet (Ellul et al. 2003a). These results indicated that the cv. Dulce Maravilla was more competent for transformation than the cv. Crimson Sweet. It also confirmed that in watermelon, as in other horticultural species such as melon (Bordas et al. 1996) and tomato (Ellul et al. 2003b), the transformation efficiency is highly cultivar dependent.

In *Citrullus colocynthis*, Dabauza et al. (1997) described a transformation efficiency of 14%. Wild watermelon was also transformed (Akashi et al. 2005) with *Agrobacterium* carrying a plasmid containing the selectable marker genes *nptII* and *hpt* (hygromycin phosphotransferase) and the reporter gene *gusA*. A GUS histochemical assay showed that kanamycin was more effective than hygromycin as the selective agent for transformation. The presence of T-DNA in the regenerated shoots was confirmed by amplification of the transgene using the polymerase chain reaction, and Southern blot analysis revealed a stable integration of the transgene in the T1 progeny. Altogether, these results indicated that both cultivated and wild species of *Citrullus* can be transformed by *A. tumefaciens*.





**Fig. 6.** Plant regeneration and genetic transformation of watermelon cv. Dulce Maravilla. **A** Half-cotyledonary explants treated the same as in all the transformation experiments but without *Agrobacterium* inoculation and cultivated on callus induction medium (CIM) without selection pressure. **B** Green kanamycin-resistant callus with shoot clusters formed on the basal cut edge of the cotyledon (CIM supplemented with kanamycin). **C** Individual transgenic shoots grown on the elongation medium without kanamycin. **D** Kanamycin-resistant shoot rooted on the selective rooting medium. **E** Transgenic plant acclimatized under greenhouse conditions. **F** Transgenic watermelon fruits. **G** Salinity tolerance test on primary shoots of R1 transgenic watermelon expressing the HAL1 gene (*left*) and control plant (*right*) cultured on rooting medium supplemented with 100 mM NaCl. **H** In vivo evaluation of homozygous transgenic lines for the HAL 1 gene and their respective azygous lines. Plants watered with 50 mM NaCl ( $EC = 6 \text{ dSm}^{-1}$ ) during 40 days under greenhouse conditions

As described in regeneration experiments by Dong and Jia (1991) and Compton and Gray (1993), the drastic loss of organogenic competence with increasing age of the donor seedlings is a major drawback in a transformation protocol. Organogenic kanamycin-resistant calli are mainly obtained from 2- to 3-day-old seedlings. However, regeneration of transgenic watermelon has also been reported from 5- and 7- to 8-day-old cotyledons of cv. Sweet Gem and cv. Melitopolski, respectively (Srivastava et al. 1991; Choi et al. 1994), but neither transformation efficiency nor inheritance and expression of the transgenes in progenies of transgenic plants were reported.

Biolistic methods have also been presented as an interesting alternative to *Agrobacterium*-mediated transformation in watermelon. Compton et al. (1993) described parameters for optimizing transient expression of a GUS gene in cotyledons following particle bombardment using a particle inflow gun (PIG). While optimum transient expression of GUS was reported, stable integration of recombinant DNA using this method was not obtained (Compton et al. 2004). According to these authors, the best method of genetically engineering watermelon may involve a combination of biolistics and *Agrobacterium*-mediated transformation. They observed that transient expression of GUS was improved when cotyledons were wounded with 1.1  $\mu\text{m}$  tungsten particles delivered using a PIG, immediately before inoculation with *Agrobacterium*. From 16 explants in each plate that were wounded using biolistics, 400–1000 GUS-expressing foci were observed. These values were 1.6- to 2.7-fold more than ones for the same number of explants wounded using a blunt scalpel before inoculation with *A. tumefaciens*, or for cotyledons bombarded with tungsten particles of similar size coated with plasmid DNA. However, stable integration of recombinant DNA or regeneration of transgenic plants was not reported (Compton et al. 2004).

## 5.2 Determination of the Ploidy of the Primary Transformants

According to Ellul et al. (2003a), the use of very young cotyledons (less than 3 days old) might explain the absence of polyploid regenerated plants. When flow cytometric analysis of nuclear DNA contents are performed on 3-day-old cotyledonary tissue of watermelon plantlets, 79% of the nuclei have 2C DNA contents (corresponding to the G1 phase of the diploid cells), 20% of the nuclei revealed 4C values (indicative of the G2/M of the diploid and the G1 of the tetraploid cells, respectively) and only 1% of the nuclei showed 8C peak, which may be due to the presence of a reduced number of tetraploid cells in the G2/M phase of the cycle.

Apart from the age of the source of explant, the regeneration and/or transformation protocol could influence the rate of change in ploidy. Recently, numerical changes in ploidy have been observed in regenerants and transgenic plants of cv. Dulce Maravilla obtained using different regeneration protocols. These results indicated that 90% of the transgenic plants and 15% of the

somaclones were tetraploids, suggesting that the regeneration of tetraploid transgenic plants in watermelon might be procedure-dependent. A similar result was obtained in genetic transformation experiments of tomato, where the rate of tetraploid transgenic plants was 20–80%, depending on both genotype and transformation procedure (Ellul et al. 2003b).

Morphological abnormalities and changes in ploidy have been reported frequently in somaclones in both melon (Moreno and Roig 1990) and watermelon (Compton et al. 1996) and transgenic plants of cucurbit species, such as cucumber (Trulson et al. 1986) and melon (Gonsalves et al. 1992). The results obtained in watermelon transformation experiments emphasize the necessity to check the ploidy of the plants before introducing transgenic material into a breeding programme. Furthermore, it indicated that, when designing a transformation protocol for breeding purposes, attention must be paid to the genetic stability of the transgenic plants.

### 5.3 Improving Watermelon Resistance to Biotic Stress

Susceptibility to a number of bacterial, fungal and viral diseases makes watermelon an excellent candidate for improvement through genetic engineering (Compton et al. 2004). While traditional breeding has resulted in cultivars resistant to anthracnose, fusarium wilt and gummy stem blight (Mohr 1986; Crall et al. 1994), the development has been difficult for plants resistant to important bacterial diseases, such as watermelon fruit blotch (Rane and Latin 1992), and viruses, such as papaya ringspot virus (PRSV; formerly known as watermelon mosaic virus-1, WMV-1) (Fehér 1993), watermelon mosaic virus (WMV; formerly referred to as watermelon mosaic virus-2, WMV-2) (Gillaspie and Wright 1993) and zucchini yellow mosaic virus (ZYMV) (Boyhan et al. 1992). Expression of bacterial, fungal and virus resistance genes through recombinant DNA technology would facilitate the development of new disease-resistant genotypes without significantly altering the genetic composition and desirable phenotypic qualities of accepted cultivars.

#### 5.3.1 *Improving Watermelon Resistance to Virus*

Since the concept of pathogen-derived resistance (PDR) has been described (Sanford and Johnston 1985), a variety of procedures have been developed to generate virus-resistant plants using genetic engineering. Since the first report that transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic tobamovirus (TMV) were protected from infections by TMV (Powell-Abel et al. 1986), pathogen-derived resistance to numerous viruses has been applied to a range of host plants, including agronomically important cucurbits. In cucumber (Gonsalves et al. 1992), squash (Fuchs and Gonsalves 1995; Tricoli et al. 1995; Fuchs et al. 1999) and melon (Fuchs et al. 1997), the expression of CP genes has been effective for controlling viral diseases under

field conditions. 'Freedom II', a yellow crookneck squash F1 hybrid cultivar, was the first virus-resistant transgenic crop plant commercially released in the USA and is described as highly resistant to single and mixed infections by ZYMV and/or WMV-2 (Fuchs and Gonsalves 1995; Tricoli et al. 1995). The transgenic squash line CZW-3 has also been reported to be resistant to ZYMV, WMV-2 and cucumber mosaic cucumovirus (CMV) (Tricoli et al. 1995).

In watermelon, resistant plants have been obtained using genetic engineering techniques based on the introduction of viral CP genes. Thus, resistance to WMV-2 was obtained in transgenic plants expressing the viral coat protein. These plants can delay the disease infection and reduce incidence and symptom expression (Wang et al. 2003). Other transgenic watermelon lines have been developed by several commercial companies and were field tested for a number of times, predominantly in the USA. However, much important data have been classified as proprietary information and were not released into the public domain. The Information System for Biotechnology (<http://www.isb.vt.edu>) indicated that the majority of the field tests for transgenic watermelon in the USA were performed for virus resistance [with plants bearing coat protein genes of both ZYMV and PRSV (Syngenta, APhi no. 01-017-13N), WMV-2 and CMV (Novartis Seed, APhi nos 00-262-11N and 00-020-01N), WMV-2 and ZYMV (Seminis Vegetable Seeds, APhi no. 98-084-20N), WMV-2 and ZYMV (Asgrow, APhi no. 95-059-02R) and WMV-2 and ZYMV (Upjohn, APhi nos 94-348-01R, 94-326-05R, 94-055-03R)]. Additionally, in 1997, a field test was conducted by Seminis Vegetable Seeds on watermelon plants bearing four coat protein genes from CMV, PRSV, WMV-2 and ZYMV (APhi no. 97-043-01R). In Europe, the only release of transgenic watermelon was reported in Italy in 1999 by Sementi Nunhems to test an increased yield trait (notification no. B/IT/99/22, <http://biotech.jrc.it/deliberate/it.asp>). Obviously, all the details of the constructs used in these works are classified as CBI (Confidential Business Information).

Researchers at Seminis Vegetable Seeds Inc. (Saticov, California) modified the protocol developed by Choi et al. (1994) to produce virus-resistant transgenic watermelon plants expressing the coat protein genes of ZYMV and WMV under control of the CaMV 35S promoter (Tricoli et al. 2002). The results indicated that transgenic inbred lines resistant to both viruses were obtained in field tests. However, data on the yield of transgenic and control plants were not presented.

As reported by Prins (2001), shortly after the concept of coat-protein-mediated resistance (CPMR) was introduced and applied against a number of economically important viruses, it was soon succeeded by alternative strategies, such as replicase-mediated resistance and movement-protein-mediated resistance. In general, these strategies produced highly variable levels of resistance and the range of viruses targeted in individual experiments varied. Trivalent transgenic watermelon plants resistant to virus infection have been created by using a trivalent vector containing genes of WMV-2 CP, and replicase genes of ZYMV and CMV. The transgenic plants were evaluated by mechanical

inoculation in the glasshouse and by field trials, and, as expected, the transgenic watermelon lines showed different phenotypes of susceptible, resistant or immune to or recovery from virus infections (Niu et al. 2005)

### 5.3.2 Improving Watermelon Fruit Blotch Tolerance

Watermelon fruit blotch (WFB) is a devastating disease caused by *Acidovorax avenae* subsp. *citrulli* (Rane and Latin 1992). Symptoms include water-soaked lesions on cotyledons and leaves as well as cracking and rotting of the fruit. The pathogen is naturally borne and transmitted by infected seeds or transplants which usually serve as the primary inoculum source. Contaminated seed results in infected seedlings with characteristic lesions on cotyledons. The disease is spread in individual fields when infected plant parts are splashed with water from precipitation or irrigation (Hopkins et al. 1993). Warm wet weather enables the disease to develop quickly, resulting in 100% infection from a few primary infection sites in a field. Fruit blotch is generally thought to become established in new areas by transmission of infected seed (Sowell and Schaad 1979; Rane and Latin 1992). Its destructive potential is explained by the fact that, under favorable conditions, it spreads quickly throughout greenhouses and in the field to infect watermelon fruit. More recently, *A. avenae* subsp. *citrulli* has been observed to cause disease on other cucurbit hosts, including cantaloupe (*Cucumis melo* L. var. *cantalupensis* Naudin), honeydew (*Cucumis melo* L.), citron [*Citrullus lanatus* (Thunb.) Matsum. and Nakai var. *citroides* (Bailey)] and pumpkin (*Cucurbita pepo* L.) (Isakeit et al. 1997, 1998; Langston et al. 1999). Chemical control of WFB has been ineffective (Rane and Latin 1992) and the most feasible disease-management strategy has been to eliminate infested seed lots prior to planting. Unfortunately, the strategy currently used is limited by lack of sensitivity and specificity, large glasshouse space and time requirements, and its destructive nature. As cultural and chemical measures are not enough to control the disease (Compton et al. 2004), genetically engineered resistance is an alternative way to generate crops able to resist pathogen infections.

In recent years, many antimicrobial peptides (AMPs) with inhibitory activities against a spectrum of microorganisms have been identified in plants (Broekaert et al. 1997) and animals (Rao 1995). Different watermelon cultivars, all of them particularly susceptible to WFB, are amenable to genetic engineering. As part of a program to develop transgenic watermelon cultivars with increased WRB resistance, the antibacterial activity of AMPs (cecropins, purothionins, puroindolines, lysozymes) has to be established against plant pathogenic bacteria (i.e., *A. avenae* subsp. *citrulli*).

The results of the bioassay conducted with these peptides and several pathogens, including *Colletotrichum acutatum* Simmonds, indicate that cecropins (Van Hoftsen et al. 1985) or magainins (Zaslloff 1987) genes may be useful for controlling bacterial fruit blotch caused by *A. avenae* subsp. *citrulli*.



Interest in the utilization of AMPs for the protection of economically important plants has led to the generation of transgenic plants expressing cecropins and their antimicrobial effect has been demonstrated in several crops (Jaynes et al. 1993; Huang et al. 1997; Osusky et al. 2000; Li et al. 2001). Magainins, a group of short peptides originally isolated from skin secretions of the African clawed frog *Xenopus laevis* and thought to function as a natural defense mechanism against microbial infections, have also been used for their antimicrobial properties in transgenic plants (DeGray et al. 2001; Li et al. 2001; Chakrabarti et al. 2003).

The examples of transgenic plants expressing genes that encode for AMP provide evidence that bacterial resistance through expression of recombinant genes is conferred in transgenic plants and could provide hope for breeding resistance to WFB disease. Field experiments will be necessary to assess the agronomic performance of watermelon transgenic lines under natural conditions and to investigate the role of transiently affected bacteria on plant growth and health. In addition, further research would determine whether other microbial groups and, in particular, plant growth promoting bacteria, could be affected by this strategy.

Another strategy could be to focus on the antimicrobial compounds released during the systemic acquired resistance (SAR; Ross 1961; Sticher et al. 1997) and the hypersensitive response (HR; Agrios 1988). SAR is an inducible defense response that is triggered in the plant by previous exposure to pathogens that cause cell death. A more rapid defense response that precedes the onset of SAR is the HR, which is localized at the site of attempted pathogen entry. HR is characterized by programmed death of host cells and is a consequence of an exquisitely specific recognition event thought to be mediated via the direct or indirect interaction between the product of a pathogen avirulence (avr) gene and the corresponding plant disease resistance (R) gene product. Highly correlated with the HR and the SAR is the production of antimicrobial compounds, the increased expression of a subset of the pathogenesis-related (PR) proteins, and the reinforcement of previously established physical/chemical barriers (Sticher et al. 1997). One of the most rapid defense responses following pathogen recognition is the so-called oxidative burst, which constitutes the production of reactive oxygen intermediates (ROIs), primarily superoxide ( $O_2^-$ ) and  $H_2O_2$ , at the site of attempted invasion (Apostol et al. 1989; Baker and Orlandi 1995). The emerging evidence suggests that the oxidative burst and cognate redox signaling may play a central role in the integration of a diverse array of plant defense responses (Grant and Loake 2000). In transgenic tomato, Coego et al. (2005) showed that the sole inhibition of the Ep5C gene expression, which encodes a secreted cationic peroxidase, was sufficient to confer resistance against the pathogen *Pseudomonas syringae* pv. *tomato*. The authors reported that these findings contribute to delineate a signaling pathway that uses early signals generated during the oxidative burst, such as  $H_2O_2$ , for the selective activation of host factors required for mounting compatible interaction and not necessarily related to the defense response (Vera and Co-

ego 2006). Thus, Ep5C could provide a new resource for developing bacterial disease-resistant watermelon varieties.

#### 5.4 Improving Salt Tolerance in Watermelon Plants

Salinity is one of the major abiotic stresses that adversely affects crop productivity and quality. About 20% of irrigated agricultural land is adversely affected by salinity (Flowers and Yeo 1995). The problem of salinity is increasing because of the use of poor quality water for irrigation and poor drainage (Chinnusamy et al. 2005). Irrigation, currently practised on about 15% of the world's farmland, produces more than one-third of the world's crops but often leaves soils with high salinity. Increased demand for clean water for urban use not only decreases the amount available for agriculture but also requires that less suitable, often moderately saline, water serves as a replacement. In addition, replacing perennial native plants, which evaporate most of the rainwater, with crops that grow only part of the year can lead to a rise in saline groundwater. Flooding, incursion of sea water into coastal fresh water reservoirs, and erratic weather patterns, which seem to increase and which add drought years, compound the effects of increasing soil salinity (Zhu et al. 2005).

According to the USDA salinity laboratory, saline soil can be defined as soil having an electrical conductivity (EC) of the saturated paste extract of  $4 \text{ dS m}^{-1}$  ( $\sim 40 \text{ mM NaCl}$ ) or more. Most grain crops and vegetables are glycophytes and are highly susceptible to soil salinity even when the soil EC is  $<4 \text{ dS m}^{-1}$  (Chinnusamy et al. 2005). Since NaCl is the major component of most saline soils, usage of the terms salinity and salt stress here refer to stress caused by high levels of NaCl. High salt causes several types of plant stress, including altered nutrient uptake, especially of ions such as  $\text{K}^+$  and  $\text{Ca}^{2+}$ , accumulation of toxic ions (mainly  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{SO}_4^-$ ) and osmotic stress. Metabolic imbalances caused by ionic toxicity, osmotic stress and nutritional deficiency under salinity may also lead to oxidative stress (reviewed in Zhu 2001, 2002).

It is generally accepted that maintaining a low cytosolic  $\text{Na}^+$  concentration is essential to promote salt tolerance and can be achieved by restricting inflow, increasing outflow or increasing vacuole sequestration of  $\text{Na}^+$  (Zhang et al. 2004a). Intuitively, increasing plasma membrane  $\text{Na}^+$  exporters and tonoplast  $\text{Na}^+$  importers and/or restricting the amount of  $\text{Na}^+$  influx by lowering the amount of plasma membrane  $\text{Na}^+$  importers should be different useful strategies. Indeed, success has been reported when these strategies have been used (Zhang et al. 2004a; Chinnusamy et al. 2005). For example, increased expression of the *Arabidopsis* tonoplast membrane  $\text{Na}^+/\text{H}^+$  antiporter, AtNHX1, under a strong constitutive promoter resulted in salt-tolerant *Arabidopsis* (Apse et al. 1999), *Brassica napus* (Zhang et al. 2001) and tomato (Zhang and Blumwald 2001).

In a similar way, overexpression of the *Saccharomyces cerevisiae* HAL1 gene (Gaxiola et al. 1992) conferred salt tolerance in this yeast by increasing intra-



cellular  $K^+$  and decreasing  $Na^+$  levels (Serrano 1996; Rios et al. 1997). In higher plants, transgenic melon ( $T_0$ ) and tomato ( $T_1$ ) overexpressing the HAL1 gene showed increased salt tolerance in vitro (Bordas et al. 1996; Gisbert et al. 2000). HAL1 also minimized the reduction in tomato fruit production caused by salt stress under glasshouse conditions (Rus et al. 2001). In watermelon, transgenic plants have been produced expressing the HAL1 gene under control of the 35S promoter with a double enhancer sequence from the cauliflower mosaic virus and the RNA4 leader sequence of the alfalfa mosaic virus (Ellul et al. 2003a). The constitutive expression of the HAL1 gene showed a beneficial effect on rooting (Fig. 6G) as well as on the development of the aerial part of  $T_1$  plants grown under in vitro saline conditions (100 mM NaCl). Moreover,  $T_2$  watermelon plants expressing the HAL1 salt-related gene always outperformed non-transformed plants under glasshouse salt-stress conditions (Fig. 6H). Without salt stress, no significant differences were observed between azygous (control) and transgenic lines. However, after 20 days of treatment with 50 mM NaCl ( $EC = 6 \text{ dS m}^{-1}$ ), growth of plants from azygous lines was severely inhibited, and significant differences were observed between control and transgenic plants at 30 and 40 days of culture. Stem elongation and the production of secondary shoots of salt-treated transgenic plants were significantly greater than those of the non-transgenic plants, indicating watermelon  $T_2$  plants carrying the HAL1 gene that had an increased ability to tolerate salinity compared with their azygous  $T_2$  controls (Ellul et al. 2003a).

Other transgenic approaches, such as manipulation of ion homeostasis, osmoprotectant accumulation, late embryogenesis abundant (LEA) proteins and radical oxygen scavenging (ROS) capacity, have demonstrated the capabilities of engineering salt-tolerant crops (Zhang et al. 2004a; Chinnusamy et al. 2005). However, few studies have been done in order to produce watermelon salt-tolerant plants. Considering that significant increases in salt tolerance can be achieved by single gene manipulation as revealed by SOS1 (Shi et al. 2003), NHX1 (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001) and HAL1 (Rus et al. 2001; Ellul et al. 2003a), and taking into account the availability of reliable and efficient transformation protocols (Choi et al. 1994; Ellul et al. 2003a), transgenic watermelon plants capable of growing, flowering and producing fruits at NaCl concentrations up to 200 mM will soon be a reality.

Although genes coming from salt-sensitive or glycophyte organisms (*Arabidopsis*, tomato or rice) can impart halotolerance (the adaptation of living organisms to conditions of high salinity), there are probably better allelic versions in nature that would result in even greater halotolerance (Zhu et al. 2005). One obvious place to search for such genetic variants is in halophyte plants (naturally growing in salinity conditions), which may carry homologue genes better suited for halotolerance. Among these species are the extremely salt-tolerant *Thellungiella halophila* (a close relative of *Arabidopsis thaliana*) and *Lycopersicon cheesmanii* (a close relative of tomato).

### 5.5 Introducing Parthenocarpy and Seedless Traits

The trait of parthenocarpy is particularly important for crop plants whose commercial product is their fruit (Varoquaux et al. 2000; Spena and Rotino 2001). During flowering, adverse environmental conditions may either prevent or reduce pollination and fertilization, decreasing fruit yield and quality. Moreover, parthenocarpic fruits are seedless, and seedlessness is highly valued by consumers in watermelon as well as in several other fruits (e.g. table grape, citrus, eggplant, cucumber). Parthenocarpic fruits have been produced by traditional breeding methods based on either mutant lines or other strategies, such as alteration of ploidy (see Sect. 4.1).

Several methods to genetically engineer parthenocarpic fruit development have been proposed, and some have also been tested experimentally in crop plants (Varoquaux et al. 2000; Spena and Rotino 2001). Transgenic parthenocarpic plants have been obtained for several horticultural crops. In particular, the chimeric gene DefH9-iaaM has been used to drive parthenocarpic fruit development in species such as eggplant (Donzella et al. 2000), tomato (Ficcardenti et al. 1999; Pandolfini et al. 2002), strawberry and raspberry (Mezzetti et al. 2004). In those experiments, the DefH9-iaaM transgene promotes the synthesis of auxin (IAA) specifically in the placenta, ovules and tissues derived therefrom (Rotino et al. 1996, 1997). The agronomic advantages of DefH9-iaaM transgenic plants have been assessed by glasshouse and field trials of DefH9-iaaM eggplant (Donzella et al. 2000; Acciarri et al. 2002), tomato (Pandolfini et al. 2002; Rotino et al. 2005), strawberry and raspberry (Mezzetti et al. 2004). In tomato, Carmi et al. (2003) tested an alternative approach for the induction of parthenocarpy that is based on ovary-specific expression of the *Agrobacterium rhizogenes*-derived gene rolB. Tomato plants transformed with a chimeric construct containing the rolB gene fused to the ovary- and young-fruit-specific promoter TPRP-F1 developed parthenocarpic fruits. Fruit size and morphology, including jelly fill in the locules of the seedless fruits, were comparable to those of seeded fruits of the parental line. Parthenocarpic fruits have also been observed in transgenic tomato plants expressing the PsEND1 promoter region to the cytotoxic barnase gene to produce male-sterile plants (Roque et al. 2004).

Search results found in the US database of field test release permits ([www.isb.vt.edu](http://www.isb.vt.edu)) indicate that transgenic watermelon has also been engineered by Seminis Vegetable Seeds to produce parthenocarpic seedless fruit. Although transgenic plants have been field tested during the last 3 years (APHIS nos. 04-064-16N, 03-091-14N, 02-064-07N), no transgenic watermelon crop has been yet released for sale (Compton et al. 2004).

## 5.6 Androsterility and Watermelon Hybrid Seed Production

Heterosis has played an important role in watermelon production, yet hybrid seed production in most countries still follows a complicated procedure of emasculating, bagging and pollinating by hand (Huang et al. 1998). The procedure is troublesome and time-consuming and it is difficult to guarantee the purity of watermelon hybrid seeds.

In this context, the use of male sterility (i.e., androsterility) seems to be the best way to solve the problem. Watts (1962) first obtained a male-sterile watermelon by irradiation and, more recently, Xia et al. (1988) found a naturally occurring male-sterile watermelon mutant. The use of this dwarf male-sterile watermelon enabled varieties to be developed that have been released, and should simplify the production of hybrid seeds and, thus, lower their cost.

In a different way, the J.P Beltrán group from the IBMCP of Valencia (Spain) characterized the promoter region of the pea *END1* gene to test its potential use as a biotechnological tool in order to specifically direct foreign gene expression to anther tissues. *END1* displays specific expression in the epidermis, connective, endothecium and middle layer cells from very early stages of anther development. In order to test the functionality of this promoter in other plant species, a construct with this promoter driving the *uidA* (GUS) gene was introduced in transgenic tomato plants which showed early specific GUS expression in anther tissues (Gomez et al. 2004). A cytotoxic gene (barnase) under the control of *END1* promoter was used with the aim of developing a genetically engineered male sterility control system. The promoter of the pea *END1* gene drove gene expression specifically to the anther and the pea *END1* promoter was fully functional in plants belonging to different families, such as the Brassicaceae (*Arabidopsis* and oilseed rape) and Solanaceae (tobacco and tomato). Male-sterile transgenic *pEND1::BARNASE* plants were regenerated. The transgenic flowers produced anther-like structures and mature pollen grains were never observed, while female fertility was unaffected (Roque et al. 2006). Male fertility could be partially restored in the F1 progeny of the cross of *pEND1::BARNASE* plants with *pEND1::BARSTAR* plants. This broad functionality, together with its spatial (structural tissues of anthers) and temporal (from stamen primordia initiation until anther dehiscence) pattern of expression, makes the *pEND1* a powerful biotechnological tool to obtain male sterile plants and to produce hybrid seeds in different plant species, for example, watermelon.

## 6 Production of Haploid and Doubled Haploid Watermelon Plants

The haploidization technique is of considerable importance as it saves time and increases efficiency in plant breeding programs. In addition, the technique pos-

sesses certain advantages in physiological and genetic studies. Ovule-ovarium culture, anther culture, interspecific hybridization and/or induction by deficient pollen are used to produce haploid plants (Allard 1960).

In cucurbitaceous plants, one of the most successful techniques is pollination by irradiated pollen to produce haploid progeny. This technique was first described in 1987 and haploid plants (H) were produced in melon (Sauton and Dumas de Vaulx 1987; Sauton 1988). Use of the technique in applied breeding programs has been limited by the low percentage of haploid embryos induced, difficulty in detecting and excising these embryos, and the limited numbers of haploid plants recovered (Lofti et al. 2003). The technique was improved by Savin et al. (1988) and Sauton (1989) using a soft X-ray technique to detect parthenogenetic embryos within seeds, and the induction of gynogenetic haploid plants in melon by using irradiated pollen became an applicable method in plant breeding. Using a similar approach, Lofti et al. (2003) developed an improved procedure for recovery of haploid (H) and doubled haploid (DH) melon plants, by culturing the seeds formed after pollination with irradiated pollen in liquid medium, before rescue of the embryos for further culture. This step made it easier to identify the seeds containing parthenogenetic embryos, thereby reducing the effort required and increasing the percentage of plants recovered (Lofti et al. 2003). The same method was applied in cucumber (Niemirowicz-Szczyt and Dumas de Vaulx 1989; Sauton 1989; Caglar and Abak 1997) and squash (Kurtar et al. 2002). An efficient gynogenetic protocol has also been described for cucumber doubled haploids production (Dirks 1995).

In watermelon, by applying the gynogenesis induced by irradiated pollen approach to the cultivars Crimson Sweet, Halep Karasi, Sugar Baby and Panonia F1, Gürsöz et al. (1991) and Sari et al. (1994) reported the rescuing of 761 embryos from 13,844 seeds. Only 17 haploid plants were obtained and doubled haploid lines were generated after chromosome doubling by immersion of in vitro cultured plants in colchicine. The low percentage of regeneration observed was probably because most of the embryos were in the globular stage and most of the heart-shaped embryos were soft or necrotic.

Although the production of haploid plants through culture techniques has become an important breeding tool, there are often difficulties in doubling the chromosome numbers of haploid plants. Chromosome doubling may occur spontaneously or may be induced by treating haploid plants with an antimitotic agent, such as colchicine either in vitro or after plants have been removed from in vitro conditions. Immersion of in vitro grown plants or their cuttings in colchicine solution has been reported in watermelon (Gürsöz et al. 1991), melon (Sauton and Dumas de Vaulx 1987; Yetisir and Sari 2003) and cucumber (Caglar and Abak 1997). Spontaneous diploidization, however, occurs only at low frequency and treatment with the toxic chemical colchicine is difficult, requires relatively large amounts of the chemical, and may result in stunting and delay in flowering, mutagenesis, ploidy chimeras and poor seed set (Herman 1997). Moreover, inbreeding effect or inbreeding depression could appear when DH plants are compared with the original diploid as, for example, in sunflower

(Tuberosa 1983), tobacco (Deaton et al. 1986; Nielsen and Collins 1989) or rice (Zhi-Kang et al. 2001). Surprisingly, in watermelon, Sari et al. (1998) did not report significant differences between original diploids and DH lines. A similar result was observed in melon by comparing 46 doubled haploid lines produced using the irradiated pollen technique with 10 original diploid genotypes (Sari and Yetisir 2002).

## 7 DNA Markers in Watermelon Breeding

Genetic markers show genetic differences between individual organisms or species. There are different types of genetic markers, generally classified as (1) morphological markers, showing differences in phenotypic traits or characteristics, (2) biochemical markers, including allelic variant of isozymes, and (3) DNA markers which reveal variation in DNA sequences. Morphological and biochemical markers are limited in number and are influenced by environmental conditions, or the developmental stage of the plant. However, independently of those limitations, they have been used for plant breeding in different species, including watermelon. In contrast, DNA makers are unlimited in number, are not affected by environmental conditions or developmental stage of the plant and have a neutral effect because they can be located in non-coding regions of the genome.

There are several DNA marker types (e.g. RAPD, AFLP, SSR, ISSR), which may be classified broadly into three groups based on their detection method: enzyme restriction with hybridization, amplification by polymerase chain reaction (PCR) and a mixture of both methods. For more information and details about molecular maker techniques and their application in plant breeding, the reader is referred to specific reviews (Dekkers and Hospital 2002; Rafalski 2002; Lörz and Wenzel 2004). In watermelon, the DNA markers could be used for construction of genetic maps, marker assisted selection, evaluation of genetic diversity and variety identification.

### 7.1 The Genetic Map in Watermelon

High-density genetic maps are useful in breeding programs for a large number of crop plants. They are also useful for positional cloning of genes and elucidating the genetic basis of complex traits that do not display Mendelian segregation (Lee 1995). Extensive genetic linkage maps have been constructed for major cucurbit species, such as melon (Baudracco-Arnas and Pitrat 1996; Wang et al. 1997; Brotman et al. 2000; Oliver et al. 2000, 2001; Périn et al. 2002) and cucumber (Park et al. 2000; Staub and Serquen 2000). However, only a few small linkage maps have been constructed for watermelon (Navot and Zamir 1986; Navot et al. 1990; Hashizume et al. 1996; Xu et al. 2000; Hawkins et al. 2001).

The first linkage map of watermelon was established in a segregating population of *C. lanatus* × *C. colocynthis* (Navot and Zamir 1986). This linkage map has 24 loci (22 isozymes, the locus for fruit bitterness and the locus for flesh color), in 7 linkage groups and 354 cM. Using a BC1 (backcross) population of an inbred line and a wild accession and RAPD (random amplified polymorphic DNA) markers, Hashizume et al. (1996) constructed a linkage map of 11 linkage groups (corresponding to the basic haploid number of chromosomes in *C. lanatus*) that covers 524 cM. However, the linkage maps included groups with only 2 RAPD markers and 12 loci were unlinked, so significant parts of the genome were not covered. Xu et al. (2000) constructed a map using an F2 population coming from a cross between a high quality inbred line and a PI number resistant to *Fusarium oxysporum* f. sp. Niveum race 0, 1 and 2. For this purpose, the authors used RAPD, SSR (simple sequence repeat), isozyme, SCAR (sequenced characterized amplified region) and morphological markers to obtain a map which covers 12,032 cM.

In the last few years, other maps have been developed using different types of populations such as test crosses with different species [(*C. lanatus* var. *citroides* × *C. lanatus* var. *lanatus*) × *C. colocynthis*], RIL (recombinant inbred lines) and different DNA markers such as RAPD, RFLP (random fragment length polymorphism) and ISSR (inter-simple sequence repeats) (Levi et al. 2002, 2004; Hashizume et al. 2003; Zhang et al. 2004b). All this information could increase the watermelon map size to 2,384 cM (Hashizume et al. 2003). The results indicate map distances comparable with those reported in melon (Baudracco-Arnas and Pitrat 1996; Wang et al. 1997; Oliver et al. 2001; Périn et al. 2002; Hashizume et al. 2003). These data are very relevant considering the interest in a comparative genetic map among different cucurbit species such as watermelon, melon, cucumber and squash. The type of marker used for the genetic map of one species could be easily transferred to the genetic map of another cucurbit species. The most interesting DNA marker to date is SSR, and several research groups are interested in developing SSR markers in Cucurbitaceae (Katzir et al. 1996; Jarret et al. 1997; Danin-Poleg et al. 2000; Levi et al. 2002; Silva et al. 2004; Joobeur et al. 2006).

Recently, Levi et al. (2006) added 114 AFLPs to the genetic linkage map previously constructed for watermelon using a test-cross population [Plant Accession no. Griffin 14113 (*C. lanatus* var. *citroides*) × New Hampshire Midget (NHM; *C. lanatus* var. *lanatus*)] × US Plant Introduction (PI) 386015 (*C. colocynthis*). This original map contained 141 RAPD markers, 27 ISSR markers and a SCAR marker that was previously reported as linked (1.6 cM) to race 1 fusarium wilt resistance in watermelon.

In melon, single-nucleotide polymorphisms (SNP) and short insertions-deletions (indels) have been found in 34 expressed sequence tag (EST) fragments between two distantly related melon genotypes (Morales et al. 2004). In watermelon, as far as we know, neither SNPs nor indels have been described. However, the discovery of SNPs based on ESTs and a suitable system for SNP



detection will have broad potential utility in melon and watermelon genome mapping.

## 7.2 Marker Assisted Selection in Watermelon

The aim of marker assisted selection (MAS) is the identification of molecular markers tightly linked to an agronomic trait for an early selection of individuals possessing this trait. When compared with other horticultural crops, watermelon is a long crop cycle species with a low density in the field. Altogether those agronomic traits make watermelon very interesting for plant breeding with MAS. Unfortunately, in watermelon, few molecular markers have been identified for interesting agronomic traits. In comparison, in melon, molecular markers are available for fusariosis (Zheng et al. 1999; Wang et al. 2000), *Aphis gossipi* (Klinger et al. 2001) and agronomic traits such as sex expression (Noguera et al. 2005) and fruit shape (Périn et al. 2002). In watermelon, molecular markers linked to resistance to *Fusarium* (Xu et al. 2000; Hawkins et al. 2001; Levi et al. 2006) and a QTL (quantitative trait loci) explaining part of the phenotypic variance of sugar content (Hashizume et al. 2003) have been described. In this context, it is clear that strong efforts need to be made to develop new molecular markers concerning several agronomic traits in watermelon, in order to improve the efficiency of the breeding programs.

## 7.3 Evaluation of Genetic Diversity in Watermelon

Another interesting application of DNA markers is the evaluation of genetic variability in watermelon. The knowledge of this genetic diversity would allow a better understanding of the possibility of developing new hybrids, and in finding new traits such as disease-resistant genes in watermelon breeding programs.

In *Citrullus* spp., molecular markers are being used to understand the relationship between the different species of this genus. As indicated earlier, the *Citrullus* genus contains four diploid species: *C. lanatus* [which includes *C. lanatus* var. *lanatus* (cultivated watermelon) and *C. lanatus* var. *citroides* (wild watermelon)], *C. colocynthis*, *C. ecirrhosus* and *C. rehmii*. Navot and Zamir (1987) studied isozyme polymorphisms and suggested that *C. lanatus* var. *citroides* is likely to be the wild progenitor of *C. lanatus* var. *lanatus*, because most of the alleles present in *C. lanatus* var. *lanatus* are also common in *C. lanatus* var. *citroides*. Results from other studies using SSR and RAPD markers supported this hypothesis and showed more genetic diversity among *C. lanatus* var. *citroides* than in *C. lanatus* var. *lanatus* (Jarret et al. 1997; Levi et al. 2001, 2002). Concerning the relationship of these four species, the analysis of ITS (internal transcribed spacer) sequence supports the idea that *C. rehmii* is more closely related to *C. lanatus* than *C. colocynthis* is to *C. lanatus* (Jarret and Newman 2000), as suggested by Navot and Zamir (1987) using isozyme studies.



RAPD and AFLP analysis was conducted by Xu et al. (2004) in order to assess the genetic relationships among 30 accessions of watermelon representing a wide range of breeding and commercially available germplasm, including cultivated and wild accessions. A tree diagram was constructed using the complete linkage method, resulting in six groups, namely, one East-Asian group, one American group, two medial groups and two African wild groups. Each ecological group exhibited specific bands, which were useful in discriminating other groups. Results of cluster analysis based on RAPD and AFLP data were consistent with, and verified, previous classification of the germplasm.

#### **7.4 Variety Identification in Watermelon**

The registration of new commercial varieties is done by DUS testing. This test is necessary to demonstrate that the new variety is different (D), uniform (U) and stable (S) in time, in comparison with other existing varieties. For this proposal, the companies and the official registrant must perform a comparison of the new varieties with the standard ones in field trials. This test is time consuming and is also dependent on the stability of the climatic conditions. For these reasons, DNA markers are very useful for registration of new varieties (Lefebvre et al. 2001). Another utility of DNA markers for seed companies is the purity control of the seed batches. Normally, this control is done by sowing large areas, with a consequent increase in the cost of land, time and people. Thus it is possible to reduce costs by using DNA markers (Lefebvre et al. 2001). Unfortunately, for watermelon, little information is available concerning the application of DNA markers in variety identification and seed quality and /or purity control.

### **8 Conclusions and Future Prospects**

Biotechnological approaches involving tissue culture, genetic engineering and molecular biology have been useful tools to overcome the constraints of conventional breeding. In watermelon, considerable information has been provided for explant tissue culture, and several studies on plant regeneration via organogenesis and somatic embryogenesis have been described. The availability of these regeneration systems allowed the development of successful genetic engineering protocols in cultivated and wild watermelon, as *Agrobacterium*-mediated transformation is found to be reasonably efficient. Using genetic engineering, several important agronomic traits, for example, pest and disease resistance and salt tolerance, have been introduced. Currently, field trials have confirmed the valuable expression of these new traits. However, genetic engineering has not been utilized yet to its full potential, and further studies in genetic engineering relating to insect resistance, antimicrobial activity, improvement of post-harvest quality and nutrient quality, parthenocarpic fruit development and androsterility need to be carried out. Furthermore, proto-

plast culture and somatic hybridization need to be studied more extensively, in order to provide efficient tools for transferring valuable agronomic traits from wild cucurbit species to cultivated watermelon.

Although molecular markers such as RAPDs, RFLPs and AFLPs have been developed, molecular marker studies with SNPs and Indels have not yet been exploited for watermelon. More extended studies are needed to develop phylogenetic maps and useful molecular markers for disease and pest resistance, parthenocarp, tolerance to abiotic stresses and many other agronomic traits. In this respect, doubled haploidy offers speed and marker technology precision and, by combining these technologies, plant breeding can be greatly improved. The recent success in doubled haploid production in major crop species coincided with the broadening of knowledge on genetic markers, QTL analysis, ESTs, candidate genes and MAS. Genetic mapping has become routine in many species and is ongoing in watermelon; the challenge now is to go beyond map locations and to identify the controlling genes present in these regions for this agronomically important crop.

However, in watermelon, protocols for doubled haploid regeneration are not efficient (as, for example, in cucumber) and much more research needs to be undertaken to improve this technique. In this respect, the use of functional genomics provides an opportunity to study (gametic) embryogenesis on a more robust scientific basis by discovering the controlling genes. The study of expressed genes via differential display or development of expression libraries is now possible and can be targeted towards finding the genes and environments responsible for efficient embryogenesis and plant cell regeneration.

## References

- Acciarri N, Restaino F, Vitelli G, Perrone D, Zottini M, Pandolfini T, Spena A, Rotino GL (2002) Genetically modified parthenocarpic eggplants: improved fruit productivity under both greenhouse and open field cultivation. *BMC Biotechnol* 4:1–10
- Agrios GN (1988) *Plant pathology*. Academic Press, London
- Akashi K, Morikawa K, Yokota A (2005) *Agrobacterium*-mediated transformation system for the drought and excess light stress-tolerant wild watermelon (*Citrullus lanatus*). *Plant Biotechnol* 22:13–18
- Al-Ghaithi F, El-Ridi MR, Adeghate E, Amiri MH (2004) Biochemical effects of *Citrullus colocynthis* in normal and diabetic rats. *Mol Cell Biochem* 261:143–149
- Allard RW (1960) *Principles of plant breeding*. John Wiley, London, p 485
- Ammirato PV (1987) Organizational events during somatic embryogenesis. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) *Plant tissue cell culture*. Alan R. Liss, New York, pp 57–81
- Andrus CE, Seshadri VS, Grimball PC (1971) Production of seedless watermelons. *Agricultural Research Service, USDA Tech Bull* 1425
- Apostol I, Heinstein FH, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction. *Plant Physiol* 90:109–116
- Apse MP, Aharon GS, Snedden WS, Blumwald E (1999) Salt tolerance conferred by over-expression of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiport in *Arabidopsis*. *Science* 285:1256–1258

- Arnold TH, De Wet BC (1993) Plants of southern Africa: names and distribution. Mem Bot Surv S Afr 62
- Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9:229–241
- Baker CJ, Orlandi EW (1995) Active oxygen species in plant pathogenesis. Annu Rev Phytopathol 33:299–321
- Bates DM, Robinson RW (1995) Cucumbers, melons and watermelons. In: Smart J, Simmonds NW (eds) Evolution of crop plants, 2nd edn. Longman, London, pp 89–96
- Baudracco-Arnas S, Pitrat M (1996) A genetic map of melon (*Cucumis melo* L.) with RFLP, RAPD, isozyme, disease resistance and morphological markers. Theor Appl Genet 93:57–64
- Bordas M, Montesinos C, Dabauza M, Salvador A, Roig LA, Serrano R, Moreno V (1996) Transfer of the yeast salt tolerance gene HAL1 to *Cucumis melo* L. cultivars and in vitro evaluation of salts tolerance. Transgenic Res 5:1–10
- Boyhan GJ, Norton D, Jacobsen BJ, Abrahams BR (1992) Evaluation of watermelon and related germplasm for resistance to zucchini yellow mosaic virus. Plant Dis 76:251–252
- Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW, Osborn RW (1997) Antimicrobial peptides from plants. Crit Rev Plant Sci 16:297–323
- Brotman Y, Silberstein L, Kovalski I, Klingler J, Thompson G, Katzir N, Perl-Treves R (2000) Linkage groups of *Cucumis melo*, including resistance gene homologues and known genes. Acta Hort 510:441–448
- Caglar G, Abak K (1997) Obtention of haploid embryo and plants with pollination by irradiated pollens in cucumber genotypes. In: Proc Turkish 2nd Natl Hort Congr, Turkey, pp 159–162
- Carmi N, Salts Y, Dedicova B, Shabtai S, Barg R (2003) Induction of parthenocarpy in tomato via specific expression of the rolb gene in the ovary. Planta 217:726–735
- Chakrabarti A, Ganapathi TR, Mukherjee PK, Bapat VA (2003) MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. Planta 216:587–596
- Chauvin JE, Souchet C, Dantec JP, Ellisèche D (2003) Chromosome doubling of 2x *Solanum* species by oryzalin: method development and comparison with spontaneous chromosome doubling in vitro. Plant Cell Tissue Organ Cult 73:65–73
- Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and improving salt tolerance in plants. Crop Sci 45:437–448
- Choi PS, Soh WY, Kim YS, Yoo OJ, Liu JR (1994) Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumefaciens*. Plant Cell Rep 13:344–348
- Coego A, Ramirez V, Ellul P, Mayda E, Vera P (2005) The H<sub>2</sub>O<sub>2</sub>-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. Plant J 42:283–293
- Compton ME, Gray DJ (1993) Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid and tetraploid watermelon. J Am Soc Hortic Sci 29:211–213
- Compton ME, Gray DJ, Hiebert E, Lin CM (1993) Expression of the  $\beta$ -glucuronidase gene in watermelon cotyledon explants following particle bombardment or infection with *Agrobacterium tumefaciens*. HortScience 28:138
- Compton ME, Gray DJ, Elmstrom GW (1996) Identification of tetraploid regenerants from cotyledons of diploid watermelon cultured in vitro. Euphytica 87:165–172
- Compton ME, Barnett N, Gray DJ (1999) Use of fluorescein discetate (FDA) to determine ploidy of in vitro watermelon shoots. Plant Cell Tissue Organ Cult 58:199–203
- Compton ME, Gray DJ, Gab VP (2004) Use of tissue culture and biotechnology for the genetic improvement of melon. Plant Cell Tissue Organ Cult 77:231–243
- Crall JM, Elmstrom GW, McCuiston FT (1994) SSDL: a high-quality icebox watermelon breeding line resistant to fusarium wilt and anthracnose. HortScience 29:707–708
- Dabauza M, Bordás M, Salvador A, Roig LA, Moreno V (1997) Plant regeneration and *Agrobacterium*-mediated transformation of cotyledon explants of *Citrullus colocynthis* (L.) Schrad. Plant Cell Rep 16:888–892
- D'Amato F (1984) Role of polyploidy in reproductive organs and tissues. In: Johri BM (ed) Embryology of angiosperms. Springer, Berlin Heidelberg New York, pp 519–556

- Danin-Poleg Y, Reis N, Baudracco-Arnas S, Pitrat M, Staub JE, Oliver M, Arus P, de Vicente CM, Katzir N (2000) Simple sequence repeats in *Cucumis* mapping and map merging. *Genome* 43:963–974
- Deaton WR, Collins GB, Nielsen MT (1986) Vigor and variation expressed by anther-derived double haploids of burley tobacco (*Nicotiana tabacum* L.). Comparison of sexual and double haploid populations. *Euphytica* 35:33–40
- DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol* 127:852–862
- Dekkers JC, Hospital F (2002) The use of molecular genetics in the improvement of agricultural populations. *Nat Rev Genet* 3:22–32
- De Winter B (1990) A new species of *Citrullus* (Benincaseae) from the Namib Desert, Namibia. *Bothalia* 20:209–211
- Dirks R (1995) Method for the production of double-haploid cucumbers. United States Patent no. 20-02-1996. 05492827
- Dolezel J (1991) Flow cytometric analysis of nuclear DNA content in higher plants. *Phytochem Anal* 2:143–154
- Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. *Ann Bot* 95:99–110
- Dolezel J, Binarova P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol Plant* 31:113–120
- Dong JZ, Jia SR (1991) High efficiency plant regeneration from cotyledons of watermelon (*Citrullus vulgaris* Schrad.). *Plant Cell Rep* 9:559–562
- Donzella G, Spena A, Rotino GL (2000) Transgenic parthenocarpic eggplants: superior germplasm for increased winter production. *Mol Breed* 6:79–86
- Ellul P (2002) Morfogénesis in vitro y obtención de plantas transgénicas de sandía (*Citrullus lanatus* [Thunb.] Matsum. & Nakai.). PhD thesis, Universidad Politécnica de Valencia, p 329
- Ellul P, Atarés A, Ramón Pons P, Roig LA, Moreno V (1999) Análisis del nivel de ploidía en somaclones y plantas transgénicas de sandía mediante evidencias morfológicas y citometría de flujo. In: Proc 3rd Reunión de la Sociedad Española de Cultivo in Vitro de Tejidos Vegetales, Málaga, p 66
- Ellul P, García-Sogo B, Pineda B, Atarés A, Ramón Pons P, Roig LA, Moreno V (2000) Factors affecting the ploidy level in transgenic plants of tomato (*Lycopersicon esculentum* Mill.) and watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai.). In: Proc Conf New Frontiers in Plant Science and Plant Biotechnology, Toulouse, p 40 (Abstract)
- Ellul P, Rios G, Atares A, Roig LA, Serrano R, Moreno V (2003a) The expression of the *Saccharomyces cerevisiae* HAL1 gene increases salt tolerance in transgenic watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai]. *Theor Appl Genet* 107:462–469
- Ellul P, García-Sogo B, Pineda B, Rios G, Roig LA, Moreno V (2003b) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. *Theor Appl Genet* 106:231–238
- Esquinas-Alcazar JT, Gulick PJ (1983) Genetic resources of Cucurbitaceae – a global report. International Board for Plant Genetic Resources, Rome
- Fehér T (1993) Watermelon: *Citrullus lanatus* (Thunb.) Matsum. & Nakai. In: Kalloo G, Bergh BO (eds) Improvement of vegetable crops. Pergamon Press, Oxford, pp 295–311
- Ficcadenti N, Sestili S, Pandolfini T, Cirillo C, Rotino GL, Spena A (1999) Genetic engineering of parthenocarpic fruit development in tomato. *Mol Breed* 5:463–470
- Flowers TJ, Yeo AR (1995) Breeding for salinity resistance in crop plants. Where next? *Aust J Plant Physiol* 22:875–884
- Fraser PD, Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. *Progr Lipid Res* 43:228–265
- Fuchs M, Gonsalves D (1995) Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. *Bio/Technology* 13:1466–1473

- Fuchs M, McFerson JR, Tricoli DM, McMaster JR, Deng RZ, Boeshore ML, Reynolds JF, Russell PF, Quemada HD, Gonsalves D (1997) Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field. *Mol Breed* 3:279–290
- Fuchs M, Gal-On A, Raccah B, Gonsalves D (1999) Epidemiology of an aphid non-transmissible potyvirus in fields of non-transgenic and coat protein transgenic squash. *Transgenic Res* 8:429–439
- Galbraith DW (1990) Flow cytometric analysis of plant genomes. *Methods Cell Biol* 33:549–562
- Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051
- Gaxiola R, de Larrinoa IF, Villalba JM, Serrano R (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J* 11:3157–3164
- Gillaspie Jr AG, Wright JM (1993) Evaluation of *Citrullus* sp. germplasm for resistance to watermelon mosaic virus 2. *Plant Dis* 77:352–354
- Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast HAL1 gene improves salt tolerance of transgenic tomato. *Plant Physiol* 123:393–402
- Gomez MD, Beltrán JP, Cañas LA (2004) The pea END1 promoter drives anther-specific gene expression in different plant species. *Planta* 219:967–981
- Gonsalves D, Chee P, Providenti Seem R, Slightom JL (1992) Comparison of coat protein-mediated and genetically derived resistance in cucumbers to infection by cucumber mosaic virus under field conditions with natural challenge inoculations by vectors. *Bio/Technology* 10:1562–1570
- Grant JJ, Loake GJ (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol* 124:21–29
- Gray DJ, Elmstrom GW (1991) Process for the accelerated production of triploid seeds for seedless watermelon cultivars. United States Patent no. 5,007,198
- Gürsöz N, Abak K, Pitrat M, Rode JC, Dumas de Vaulx R (1991) Obtention of haploid plants induced by irradiated pollen in watermelon (*Citrullus lanatus*). *Cucurbit Genet Coop Rep* 14:109–110
- Hansen NJP, Andersen SB (1996) In vitro chromosome doubling potential of colchicine, oryzalin, trifluralin, and APM in *Brassica napus* microspore culture. *Euphytica* 88:159–164
- Hashizume T, Shimamoto I, Harushima Y, Yui M, Sato T, Imai T, Hirai M (1996) Construction of a linkage map for watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) using random amplified polymorphic DNA (RAPD). *Euphytica* 90:265–273
- Hashizume T, Shimamoto I, Hirai M (2003) Construction of a linkage map and QTL analysis of horticultural traits for watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] using RAPD, RFLP and ISSR markers. *Theor Appl Genet* 106:779–785
- Hawkins LK, Dane F, Kubisiak TL, Rhodes BB, Jarret RL (2001) Linkage mapping in a watermelon population segregating for fusarium wilt resistance. *J Am Soc Hortic Sci* 126:344–350
- Heller FO (1973) DNS-Bestimmung an Keimwurzeln von *Vicia faba* L. mit Hilfe der Impulscytotopometrie. *Ber Dsch Bot Ges* 86:437–441
- Herman E (1997) Chromosome doubling techniques. In: Recent advances in plant tissue culture, vol 5. New techniques and systems for growth, regeneration and micropropagation. *Agricell Rep* 1995–1997
- Hopkins DL, Thompson CM, Elmstrom GW (1993) Resistance of watermelon seedlings and fruit to the fruit blotch bacterium. *HortScience* 28:122–123
- Huang HX, Zhang XQ, Wei ZC, Li QH, Li X (1998) Inheritance of male-sterility and dwarfism in watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai]. *Sci Hort* 74:175–181
- Huang Y, Nordeen RO, Di M, Owens LD, McBeath JH (1997) Expression of an engineered cecropin gene cassette in transgenic tobacco plants confers resistance to *Pseudomonas syringae* pv. *tabaci*. *Phytopathology* 87:494–499
- Isakeit T, Black MC, Barnes LW, Jones JB (1997) First report of infection of honeydew with *Acidovorax avenae* subsp. *citrulli*. *Plant Dis* 81:694

- Isakeit T, Black MC, Jones JB (1998) Natural infection of citron melon with *Acidovorax avenae* subsp. *citrulli*. Plant Dis 82:351
- Jarret RL, Newman M (2000) Phylogenetic relationships among species of *Citrullus* and the placement of *C. rehmii* De Winter as determined by internal transcribed spacer (ITS) sequence heterogeneity. Genet Res Crop Evol 47:215–222
- Jarret RL, Merrick LC, Holms T, Evans J, Aradhya MK (1997) Simple sequence repeats in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai). Genome 40:433–441
- Jaworski JM, Compton ME (1997) Plant regeneration from cotyledons of five watermelon cultivars. HortScience 32:469
- Jaynes JM, Nagpala P, Destefano-Beltran L, Huang JH, Kim JH, Denney T, Cetiner S (1993) Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Sci 89:43–53
- Joobeur T, Gusmini G, Zhang X, Levi A, Xu Y, Wehner TC, Oliver M, Dean RA (2006) Construction of a watermelon BAC library and identification of SSRs anchored to melon or Arabidopsis genome. Theor Appl Genet 112:1553–1560
- Kato A (1999) Induction of bicellular pollen by trifluralin treatment and occurrence of triploids and aneuploids after fertilization in maize. Genome 42:154–157
- Katzir N, Danin-Poleg Y, Tzuri G, Karchi Z, Lavi U, Cregan PB (1996) Length polymorphism and homologies of microsatellites in several cucurbitaceae species. Theor Appl Genet 93:1282–1290
- Kermani MJ, Sarasan V, Roberts AV, Yokoya K, Wentworth J, Sieber VK (2003) Oryzalin-induced chromosome doubling in *Rosa* and its effect on plant morphology and pollen viability. Theor Appl Genet 107:1195–1200
- Kihara H (1951) Triploid watermelons. Proc Am Soc Hortic Sci 58:217–230
- Klinger J, Kovalski I, Silberstein L, Thompson GA, Perl-Treves R (2001) Mapping of cotton-melon aphid resistance in melon. J Am Soc Hortic Sci 126:56–63
- Kurtar ES, Sari N, Abak K (2002) Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica 127:335–344
- Lagemann J, Flinn JC, Okigbo BN, Moormann FR (1975) Root crops/oil palm farming systems: a case study from eastern Nigeria, Ibadan. International Institute of Tropical Agriculture Rep, Ibadan, Oyo State, Nigeria, pp 3–45
- Langston DB, Walcott RR, Gitaitis RD, Sanders FH (1999) First report of a fruit rot of pumpkin caused by *Acidovorax avenae* subsp. *citrulli* in Georgia. Plant Dis 83:199
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation: a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214
- Lee M (1995) DNA markers and plant breeding programs. Adv Agron 55:265–344
- Lefebvre V, Goffinet B, Chauvet JC, Caromel B, Signoret P, Brand R, Palloix A (2001) Evaluation of genetic distances between pepper inbred lines for cultivar protection purposes: comparison of AFLP, RAPD and phenotypic data. Theor Appl Genet 102:741–750
- Levi A, Thomas CE, Keinath AP, Wehner TC (2001) Genetic diversity among watermelon (*Citrullus lanatus* and *Citrullus colocynthis*) accessions. Genet Res Crop Evol 48:559–566
- Levi A, Thomas CE, Joobeur T, Zhang X, Davis A (2002) A genetic linkage map for watermelon derived from a testcross population: (*Citrullus lanatus* var. *citrinoides* × *C. lanatus* var. *lanatus*) × *Citrullus colocynthis*. Theor Appl Genet 105:555–563
- Levi A, Thomas CE, Newman M, Reddy OUK, Zhang X, Xu Y (2004) ISSR and AFLP markers differ among American watermelon cultivars with limited genetic diversity. J Am Soc Hortic Sci 129:553–558
- Levi A, Thomas CE, Reddy OK, Harrison Dunn ML (2006). An extend linkage map for watermelon based on SRAP, AFLP, SSR, ISSR and RAPD markers. J Am Soc Hortic Sci 131:393–402
- Li Q, Lawrence CB, Xing HY, Babbitt RA, Bass WT, Maiti IB, Everett NP (2001) Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. Planta 212:635–639



- Lofti M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003) Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. *Plant Cell Rep* 21:1121–1128
- Lörz H, Wenzel G (2004) Molecular marker systems in plant breeding and crop improvement. Springer, Berlin Heidelberg New York
- Lower RL, Johnson KW (1969) Observations on sterility of induced autotetraploid watermelons. *J Am Soc Hortic Sci* 94:367–369
- Maggs-Kölling GL, Madsen S, Christiansen JL (2000) A phenetic analysis of morphological variation in *Citrullus lanatus*. *Namibia Genet Resour Crop Evol* 47:385–393
- Mallick MFR, Masui M (1986) Origin, distribution and taxonomy of melons. *Sci Hort* 28:251–261
- Marr CW, Gast KLB (1991) Reactions by consumers in a ‘farmers’ market to prices for seedless watermelon and ratings of eating quality. *HortTechnology* 1:105–106
- McCuiston G, Elmstrom GW (1993) Identifying polyploids of various cucurbits. *Proc Fla State Hortic Soc* 106:155–157
- Meese ADJ (1962) The Cucurbitaceae of southern Africa. *Bothalia* 8:1–111
- Mezzetti B, Landi L, Pandolfini T, Spena A (2004) The *defH9-iaaM* auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. *BMC Biotechnol* 4:1–10
- Mohr HC (1986) Watermelon breeding. In: Bassett MJ (ed) *Breeding vegetable crops*. AVI, Westport, pp 37–66
- Morales M, Roig E, Monforte AJ, Arús P, Garcia-Mas J (2004) Single-nucleotide polymorphisms detected in expressed sequence tags of melon (*Cucumis melo* L.). *Genome* 47:352–360
- Morejohn LC, Bureau TE, Mole-Bajer T, Bajer AS, Fosket DE (1987) Oryzalin, a dinitro-aniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. *Planta* 172:252–264
- Moreno V, Roig LA (1990) Somaclonal variation in Cucurbits. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 2. Somaclonal variation in crop improvement 1. Springer, Berlin Heidelberg New York, pp 435–464
- Nagl W (1976) DNA endoreduplication and polyteny understood as evolutionary strategies. *Nature* 261:614–615
- Navot N, Zamir D (1986) Linkage relationships of 19 protein-coding genes in watermelon. *Theor Appl Genet* 72:274–278
- Navot N, Zamir D (1987) Isozyme and seed protein phylogeny of the genus *Citrullus* (Cucurbitaceae). *Plant Syst Evol* 156:61–67
- Navot N, Sarfatti M, Zamir D (1990) Linkage relationships of genes affecting bitterness and flesh color in watermelon. *J Hered* 81:162–165
- Nielsen MT, Collins GB (1989) Variation among androgenic and gynogenic double haploids of tobacco (*Nicotiana tabacum* L.). *Euphytica* 43:263–267
- Niemirowicz-Szcytt K, Dumas De Vaulx R (1989). Preliminary data on haploid cucumber (*Cucumis sativus* L.) induction. *Cucurbit Genet Coop Rep* 12:24–25
- Niu SN, Huang XS, Wong SM, Yu JL, Zhao FX, Li DW, Wang SY, Zhai GM, Shi FS (2005) Creation of trivalent transgenic watermelon resistant to virus infection. *J Agric Biotechnol* 13:10–15
- Noguera FJ, Capel J, Alvarez JI, Lozano R (2005) Development and mapping of a codominant SCAR marker linked to the andromonoecious gene of melon. *Theor Appl Genet* 110:714–720
- Oliver M, Garcia-Mas J, Morales M, Dolcet-Sanjuan R, Carmen de Vincente M, Gomez H, van Leewen H, Monfort A, Puigdomenech P, Arus P (2000) The Spanish melon genome project: construction of a saturated genetic map. *Acta Hort* 510:375–378
- Oliver M, Garcia-Mas J, Cardús M, Pueyo N, López-Sesé AI, Arroyo M, Gómez-Paniagua H, Arús P, de Vicente MC (2001) Construction of a reference linkage map for melon. *Genome* 44:836–845
- Osusky M, Zhou G, Osuska L, Hancock RE, Kay WW, Misra S (2000) Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. *Nat Biotechnol* 18:1162–1166



- Pandolfini T, Rotino GL, Camerini S, Defez R, Spena A (2002) Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. *BMC Biotechnol* 1:1–11
- Park YH, Sensoy S, Wye C, Antonise R, Peleman J, Havey MJ (2000) A genetic map of cucumber composed of RAPDs, RFLPs, AFLPs, and loci conditioning resistance to papaya ringspot and zucchini yellow mosaic viruses. *Genome* 43:1003–1010
- Périn C, Hagen LS, De Conto V, Katzir N, Danin-Poleg Y, Portnoy V, Baudracco-Arnas S, Chadoeuf J, Dogimont C, Pitrat M (2002) A reference map of *Cucumis melo* based on two recombinant inbred line populations. *Theor Appl Genet* 104:1017–1034
- Petersen KK, Hagberg P, Kristiansen P (2002) In vitro chromosome doubling of *Miscanthus sinensis*. *Plant Breed* 121:445–450
- Pitrat M, Chauvet M, Foury C (1999) Diversity, history and production of cultivated cucurbits. In: *Proc 1st Int Symp on Cucurbits*. *Acta Hort* 492:21–28
- Powell-Abel P, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232:738–743
- Prins M (2001) Broad virus resistance in transgenic plants. *Trends Biotechnol* 21:373–375
- Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. *Curr Opin Plant Biol* 5:94–100
- Rane KK, Latin RX (1992) Bacterial fruit blotch of watermelon: association of the pathogen with seed. *Plant Dis* 76:509–512
- Rao AG (1995) Antimicrobial peptides. *Mol Plant Microbe Interact* 8:6–13
- Reed J, Privalle L, Powell ML, Meghji M, Dawson J, Dunder E, Suttie J, Wenck A, Launis K, Kramer C, Chang YF, Hansen G, Wright M, Chang YF (2001) Phosphomannose isomerase: an efficient selectable marker for plant transformation. *In Vitro Cell Dev Biol-Plant* 37:127–132
- Rios G, Ferrando A, Serrano R (1997) Mechanism of salt tolerance conferred by over-expression of the *HAL1* gene in *Saccharomyces cerevisiae*. *Yeast* 13:515–528
- Robinson RW, Decker-Walters DS (1997) Cucurbits. CAB International, Wallingford, Oxfordshire
- Roque E, Ellul P, Gómez MD, Madueño F, Beltrán JP, Cañas LA (2004) Tomates partenocárpicos y procedimiento para su producción. Oficina Española de Patentes y Marcas P200401761. Patent form 17/07/2004
- Roque E, Gómez MD, Ellul P, Wallbraun M, Madueño F, Beltrán JP, Cañas LA (2006) The PsEND1 promoter: a novel tool to produce genetically engineered male-sterile plants by early anther-ablation. *Plant Cell Rep* (in press)
- Ross AF (1961) Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14:340–358
- Rotino GL, Sommer H, Saedler H, Spena A (1996) Methods for producing parthenocarpic or female sterile transgenic plants and methods for enhancing fruit setting and development. EPO Patent no. EPO 96120645.5
- Rotino GL, Perri E, Zottini M, Sommer H, Spena A (1997) Genetic engineering of parthenocarpic plants. *Nat Biotechnol* 15:1398–1401
- Rotino GL, Acciarri N, Sabatini E, Mennella G, Lo Scalzo R, Maestrelli A, Molesini B, Pandolfini T, Scalzo J, Mezzetti B, Spena A (2005) Open field trial of genetically modified parthenocarpic tomato: seedlessness and fruit quality. *BMC Biotechnol* 32:1–8
- Rus AM, Estan MT, Gisbert C, García-Sogo B, Serrano R, Caro M, Moreno V, Bolarin MC (2001) Expressing the yeast *HAL1* gene in tomato increases fruit yield and enhances  $K^+/Na^+$  selectivity under salt stress. *Plant Cell Environ* 24:875–880
- Sanford JC, Johnston SA (1985) The concept of pathogen-derived resistance. *J Theor Biol* 113:395–405
- Sari N, Yetisir H (2002) Some agronomical characteristics of doubled haploid lines produced by irradiated pollen technique and parental diploid genotypes in melons. *Turk J Agric For* 26:311–317
- Sari N, Abak K, Pitrat M, Rode JC, Dumas de Vaulx R (1994) Induction of parthenogenetic haploid embryos after pollination by irradiated pollen in watermelon. *HortScience* 29:1189–1190

- Sari N, Abak K, Yetisir H, Bamyacioglu O (1998) Comparison of diploid and dihaploid watermelon genotypes for yield and some other agronomic characteristics. In: Proc 2nd Symp on Vegetable Cultivation, Tokat, pp 193–198
- Sari N, Ekiz H, Yücel S, Yetisir H, Ekbic E (1999a) Investigation of new protected cultivation melon lines resistant to *Fusarium oxysporum* f. sp. *melonis* using dihaplodization. In: Proc 3rd Int Horticultural Congr, Ankara, pp 498–503
- Sari N, Abak K, Pitrat M (1999b) Comparison of ploidy level screening methods in watermelon: *Citrullus lanatus* (Thunb.) Matsum. and Nakai. Sci Hort 82:265–277
- Sauton A (1988) Effect of season and genotype on gynogenetic haploid production in muskmelon, *Cucumis melo* L. Sci Hort 35:71–75
- Sauton A (1989) Haploid gynogenesis in *Cucumis sativus* induced by irradiated pollen. Cucurbit Genet Coop Rep 12:22–23
- Sauton A, Dumas De Vaulx R (1987) Obtention de plantes haploïdes chez le melon (*Cucumis melo* L.) par gynogénèse induite par du pollen irradié. Agronomie 7:141–148
- Savin F, Decombe V, Le-Courriour M, Hallard J (1988) The X-ray detection of haploid embryos arisen in muskmelon (*Cucumis melo* L.) seeds and resulting from a parthenogenetic development induced by irradiated pollen. Cucurbit Genet Coop Rep 11:36–42
- Serrano R (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol 165:1–52
- Shi H, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter improves salt tolerance in Arabidopsis. Nat Biotechnol 21:81–85
- Silva Ritschel P, de Lima Lins TC, Tristan RL, Cortopassi Buso GS, Buso JA, Ferreira ME (2004) Development of microsatellite markers from an enriched genomic library for genetic analysis of melon (*Cucumis melo* L.). BMC Plant Biol 4:1
- Skorupska HT, Allgood NG (1990) Staining procedure for watermelon somatic chromosomes. Cucurbit Genet Coop Rep 13:47–48
- Sowell G, Schaad NW (1979) *Pseudomonas pseudoalcaligenes* subsp. *citrulli* on watermelon: seed transmission and resistance of plant introductions. Plant Dis Rep 63:437–441
- Spena A, Rotino GL (2001) Parthenocarp: state of the art. In: Bhojwani SS, Soh WY (eds) Current trends in the embryology of angiosperms. Kluwer, Dordrecht, pp 435–450
- Srivastava DK, Andrianov VM, Piruzian ES (1991) Regeneration and genetic transformation studies in watermelon (*Citrullus vulgaris* L. cv. Melitopolski). In: Prakash J, Pierik RLM (eds) Horticulture – new technologies and applications. Kluwer, Dordrecht, pp 127–130
- Staub JE, Serquen FC (2000) Towards an integrated linkage map of cucumber: map merging. Acta Hort 510:357–366
- Sticher L, Mauch-Mani B, Métraux JP (1997) Systemic acquired resistance. Annu Rev Phytopathol 35:235–270
- Sugiyama K, Morishita M (2000) Production of seedless watermelon using soft-X-irradiated pollen. Sci Hort 84:255–264
- Takamura T, Lim KB, Van Tuyl JM (2002) Effect of a new compound on the mitotic polyploidization of *Lilium longiflorum* and oriental hybrid lilies. Acta Hort 572:37–42
- Tricoli DM, Carney KJ, Russell VM, McMaster JR, Groff DW, Hadden KC, Hubbard JP, Boeshore ML, Quemada HD (1995) Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic virus, watermelon mosaic virus 2, and zucchini yellow mosaic virus. Bio/Technology 13:1458–1465
- Tricoli DM, Carney KJ, Russell PF, Quemada HD, McMaster RJ, Reynolds JF, Deng RZ (2002) Transgenic plants expressing DNA constructs containing a plurality of genes to impart virus resistance. United States Patent no. 6:337,431
- Trulson AJ, Simpson RB, Shanin EA (1986) Transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes*. Theor Appl Genet 73:11–15
- Tuberosa R (1983) Inbreeding effect in a population of sunflower (*Helianthus annuus* L.). Genet Agraria 37:411–419

- Van Hoftsen P, Faye I, Kockum K, Lee JY, Xanthopoulos KG, Boman IA, Boman HG, Engstrom A, Andreu D, Merrifield RB (1985) Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. *Proc Natl Acad Sci USA* 82:2240–2243
- Van Tuyl JM, Meijer H, Van Diën MP (1992) The use of oryzalin as an alternative for colchicine in in-vitro chromosome doubling of *Lilium* and *Nerine*. *Acta Hort* 325:625–630
- Varoquaux F, Blanvillain R, Delseny M, Gallois P (2000) Less is better: new approaches for seedless fruit production. *Trends Biotechnol* 18:233–242
- Veilleux RE, Johnson AAT (1998) Somaclonal variation: molecular analysis, transformation interaction, and utilization. *Plant Breed Rev* 16:229–268
- Vera P, Coego A (2006) Transgenic tomato plants with acquired resistance to *Pseudomonas syringae* pv. *tomato*. International Patent Publ no. WO/2006/013226. Publication date 02/09/2006. International Application no. PCT/ES2005/070112
- Wang HZ, Zhao PJ, Xu JC, Zhao H, Zhang HS (2003) Virus resistance in transgenic watermelon plants containing a WMV-2 coat protein gene. *Yi Chuan Xue Bao* 30:70–75 (in Chinese)
- Wang YN, Thomas CE, Dean RA (1997) A genetic map of melon (*Cucumis melo* L.) based on amplified fragment length polymorphism (AFLP) markers. *Theor Appl Genet* 95:791–798
- Wang YH, Thomas CE, Dean RA (2000) Genetic mapping of a fusarium wilt resistance gene (Fom-2) in melon (*Cucumis melo* L.). *Mol Breed* 6:379–389
- Watts VM (1962) A marked male-sterile mutant in watermelon. *Proc Am Soc Hortic Sci* 81:498–505
- Wester AR (1989) The flora of the United Arab Emirates: an introduction. UAE University, Al Ain
- Xia XT, Liu YA, Liu EJ, Chen AB (1988) Selection of dual-purpose male-sterile line of G17AB watermelon. *J Shenyang Agric Univ* 19 1:9–13 (in Chinese)
- Xu Y, Zhang HY, Kang GB, Wang YJ, Chen H (2000) Studies of molecular marker-assisted selection for resistance to fusarium wilt in watermelon (*Citrullus lanatus*) breeding. *Acta Genet Sin* 27:151–157
- Xu Y, Guo J, Zhang H, Gong G (2004) Analysis of genetic relationships of watermelon [*Citrullus lanatus* (Thunb.) Mansfeld] germplasm using RAPD and AFLP. *Acta Hort* 637:271–278
- Yetisir H, Sari N (2003) A new method for haploid muskmelon (*Cucumis melo* L.) dihaploidization. *Sci Hort* 98:277–283
- Zaslloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 84:5449–5453
- Zeven AC, de Wet MJM (1982) Dictionary of cultivated plants and their regions of diversity, 3rd edn. Centre for Agricultural Publishing and Documentation, Wageningen
- Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat Biotechnol* 19:765–768
- Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc Natl Acad Sci USA* 98:12832–12836
- Zhang JZ, Creelman RA, Zhu JK (2004a) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 135:615–621
- Zhang R, Xu Y, Yi K, Zhang H, Liu L, Gong G, Levi A (2004b) A genetic linkage map for watermelon derived from recombinant inbred lines. *J Am Soc Hortic Sci* 129:237–243
- Zhang XP, Rhodes BB, Whitesides JF (1994) Determination of watermelon ploidy level using flow cytometry. *Cucurbit Genet Coop Rep* 17:102–105
- Zhao J, Simmonds DH (1995) Application of trifluralin to embryogenic microspore cultures to generate doubled haploid plants in *Brassica napus*. *Physiol Plant* 95:304–309
- Zheng XY, Wolff DW, Baudracco-Arnas S, Pitrat M (1999) Development and utility of cleaved amplified polymorphic sequences (CAPS) and restriction fragment length polymorphisms (RFLPs) linked to the Fom-2 fusarium wilt resistance gene in melon (*Cucumis melo* L.). *Theor Appl Genet* 99:453–463

- Zhi-Kang L, Lou LJ, Mei HW, Wang DL, Shu QY, Tabien R, Zhong DB, Ying CS, Stansel JW, Khush GS, Paterson AH (2001) Overdominant epistatic loci are the primary genetic basis of inbreeding depression and heterosis in rice. I. Biomass and grain yield. *Genet Soc Am* 158:1737–1753
- Zhu JK (2001) Plant salt tolerance. *Trends Plant Sci* 6:66–71
- Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* 53:247–273
- Zhu JK, Bressan RA, Hasegawa PM, Pardo JM, Bohnert HJ (2005) Salt and crops: salinity tolerance. In: *Success stories in agriculture*. Council for Agricultural Science and Technology, Ames, Iowa
- Zohary D, Hopf M (1988) *Domestication of plants in the Old World*. Clarendon Press, Oxford

## I.7 Avocado

R.E. LITZ<sup>1</sup>, S.H.T. RAHARJO<sup>1</sup>, and M.A. GÓMEZ LIM<sup>2</sup>

### 1 Introduction

The family Lauraceae consists primarily of tree species, a few of which are economically important, including avocado (*Persea americana*), bay leaf (*Laurus nobilis*), cinnamon (*Cinnamomum zeylanicum*) and camphor (*Cinnamomum camphora*). The avocado is the only commercially important fruit species within the family. The genus *Persea* (Clus.) Miller possibly originated in Gondwanaland, and spread to Asia and to North America via Europe. During the Paleocene, the genus must have spread to South America via Antarctica. When the land bridge between North and South America was established during the late Neocene, the genus would have been reunited. The Southeast Asian *Machilus* Nees, *Nothaphoebe* Blume and *Alseodaphne* Nees may be congeneric with *Persea* (Kostermans 1952). Central America has been an area of extensive speciation (Scora and Bergh 1990). There are two subgenera within the genus *Persea*, namely *Eriodaphne* (South America) and *Persea* (Central America) (Kopp 1966). Scora et al. (2002) identified three species within the subgenus *Persea*, these being *P. schiedeana* Nees, *P. parviflora* Williams and *P. americana* Mill.

There are eight well-defined *P. americana* subspecies, including *P. americana* var. *nubigena* (Williams) Kopp, var. *steyermarkii* Allen, var. *zentmyerii* Schieber and Bergh, var. *floccosa* Mez. and var. *tolimanensis* Zentmyer and Schieber. The other subspecies comprise the avocado (Bergh and Ellstrand 1986), and include the Mexican subspecies *P. americana* var. *drymifolia* (Schlect. and Cham.) Blake, which is adapted to the tropical highlands, the Guatemalan subspecies *P. americana* var. *guatemalensis* Williams, which is adapted to medium elevations in the tropics, and the West Indian subspecies *P. americana* var. *americana* Mill., which is adapted to the lowland, humid tropics (Popenoe 1941).

Avocado trees are evergreen, and mature trees are approx. 20 m in height with a shallow unsuberized secondary root system; anchorage roots can penetrate to 3–4 m (Whiley 1992). Flowers are borne on the terminals of twigs as panicles of cymes. The avocado fruit is a large, fleshy pyriform or globose berry with a single seed. The species has a long juvenile period, and is very heterogeneous because of the low incidence of self-pollination. Avocado cultivars

<sup>1</sup> Tropical Research and Education Center, University of Florida, 18905 SW 280 St, Homestead, Florida 33031-3314, USA, e-mail: rel@ifas.ufl.edu

<sup>2</sup> Centro de Investigacion y de Estudios Avanzados del IPN (CINVESTAV), Apartado Postal 629, Irapuato GTO, Mexico 36500

are classified as either A or B types, due to their flowering pattern. Each flower opens twice over a 2-day period, and all open flowers are functionally either female or male. Type A cultivars have functionally female flowers in the morning, whereas flowers of type B cultivars are functionally female in the afternoon.

The world production of avocado was approx. 3,040,496 Mt in 2003 (FAO-STAT 2004, <http://apps.fao.org>), with greatest production in Mexico (1,040,390 Mt), the USA (200,000 Mt), Brazil (173,000 Mt), Indonesia (157,500 Mt), Dominican Republic (150,000 Mt), Colombia (144,000 Mt), Chile (135,000 Mt) and Spain (135,000 Mt). Avocado is consumed as a fresh fruit, and is a rich source of oil. The estimated value of avocado exports in 2002 was \$447,787,000 (FAOSTAT 2004), exports being lead by Chile, Mexico, Spain and Israel. The EU and North America are the largest markets for avocados, and the most important export cultivar is 'Hass'.

## 2 Breeding and Genetics

Advances in breeding and genetics of avocado have been reviewed by Bergh and Lahav (1996) and Lahav and Lavi (2002). Avocado breeding programmes, particularly those in California (USA) and Israel, have been relatively successful, despite the limitations imposed by the long juvenile and evaluation periods, land and labour. Even so, most of the important cultivars have been derived from open pollinations and dooryard tree selections. The chromosome number is  $2n = 2x = 24$  (Garcia 1975). It is notable that although avocado has 24 chromosomes, it has a relatively small haploid genome size, which is six times as large as the recently sequenced genome of *Arabidopsis thaliana* (the plant with the smallest genome size known), slightly smaller than that of tomato and twice as large as that of rice (Arumuganathan and Earle 1991). Little is known about the inheritance of horticultural traits in avocado. Most morphological traits are probably coded by several loci with several alleles in each locus (Lavi et al. 1993a, b). The dwarf trait of *P. schiedeana* has been attributed to a single gene (Bergh and Lahav 1996).

Many avocado cultivars are hybrids involving two or more subspecies (Guatemalan, Mexican and West Indian), e.g., 'Hass' and 'Fuerte' are probably Guatemalan  $\times$  Mexican hybrids. Early Californian avocado cultivars were selections made in Mexico and Central America, or were selected from imported seeds (Bergh 1957). 'Benik', 'Itzamna' and 'Nabal' (Guatemalan) were introduced as bud wood, whereas 'Dickinson' and 'Fuerte' originated from seed. Seedlings derived from open pollinations were selected later in California ('Bacon', 'Hass', 'Pinkerton', 'Reed', 'Zutano'), and were thereafter distributed worldwide to regions with Mediterranean and subtropical climates. Florida (USA) avocado production is based upon selections of West Indian and Guatemalan  $\times$  West Indian hybrids, which have been introduced to many tropical regions.

## 2.1 Rootstock Improvement

*Phytophthora* root rot (PRR) disease, which is caused by the soil-borne oomycete *Phytophthora cinnamomi* Rands., may well be the most significant limiting factor for avocado production (Coffey 1987; Whiley 1992; Ben-Ya'acov and Michelson 1995). Resistance to PRR does not occur in avocado and in other species of the subgenus *Persea*. However, resistance to PRR does occur in species within the subgenus *Eriodaphne*, although these species are sexually and graft-incompatible with species in the subgenus *Persea* (Zentmyer 1980; Bergh and Ellstrand 1986; Lahav and Lavi 2002).

The rootstock selections 'Duke' (together with its progeny, 'Duke 7', 'Barr-Duke' and 'D9'), 'Thomas' and 'G6' have good tolerance to PRR. However, some PRR-tolerant selections have serious limitations. The PRR-tolerant interspecific hybrid 'Martin Grande' (G755) (*P. schiedeana* × *P. americana*) (Ellstrand et al. 1986) produces trees with poor yields. Some rootstocks with tolerance to PRR have no tolerance to another serious soil-borne pathogen, *Phytophthora citricola* (Tsao et al. 1992). Rootstocks of the Guatemalan race appear to be more sensitive to *Dothiorella* and *Verticillium* wilt than Mexican race rootstocks (Zentmyer et al. 1965).

Size control is a major production cost because of the large and spreading growth habit of the avocado tree. Dwarfing rootstock could significantly impact avocado production by reducing harvesting costs from compact trees and by increasing the density of plantings, which would result in greater yields. Sánchez-Colin and Barrientos-Priego (1987) reported that 'Colin V-33', used either as an interstock or as a rootstock, could confer dwarf habit to the scion. However, the growth habit of this selection suggests that it may have been infected with avocado sunblotch viroid (ASBVd).

Much avocado production occurs where water is either saline or in short supply, necessitating the use of brackish water for irrigation, e.g., in California, Israel and Australia. Consequently, in order to optimize production, clonal rootstocks should be tolerant of saline conditions. The West Indian avocado race demonstrates the most resistance to saline conditions, while the Mexican race shows the least resistance, although significant variability occurs within each race (Kadman and Ben-Ya'acov 1976) and amongst seedlings from the same tree (Kadman 1968). Resistance to calcium-induced chlorosis is also greater in West Indian cultivars, although there is considerable variability for this trait within seedling populations (Ben-Ya'acov 1972). West Indian rootstocks generally perform poorly in heavy soils and under waterlogged conditions (Ben-Ya'acov et al. 1974).

## 2.2 Scion Improvement

The black-skinned 'Hass' and, to a lesser extent, the green-skinned 'Fuerte' represent the market standards for avocados. Optimum fruit size is about



250–350 g (Lahav and Lavi 2002), with either pyriform ('Hass') or ovate ('Bacon' and 'Gwen') shape. Peel that is easily removed is preferred. Small seed size is desirable in Mexican and Mexican  $\times$  Guatemalan types, although this trait is uncommon in West Indian avocados. Size is variable within each cultivar, and is influenced by stage of maturity, cultural practices and climatic conditions (Lahav and Kalmar 1977; Whiley and Schaffer 1994). Cultivars resembling 'Hass' have been released to complement this selection, particularly during its off season, e.g., 'Gwen', 'Jim', 'Lamb Hass' and 'Reed'.

Mexican-type avocado fruit are strongly climacteric (Adato and Gazit 1977) and a fixed climacteric phase precedes a variable lag phase (Eaks 1980). Low concentrations of endogenous ethylene are produced during the lag phase, which triggers the climacteric as sensitivity to ethylene increases (McMurchie et al. 1972). Ripening cannot be arrested after it has been initiated. Fruit of Guatemalan and Mexican cultivars and their hybrids can be stored on the tree during the lag phase for 2–4 months after reaching maturity (Whiley 1992); ripening occurs only after the fruit have been harvested. West Indian and West Indian  $\times$  Guatemalan cultivars cannot be stored on the tree in this manner, and must be picked at maturity. On-tree storage, together with production in different climatic conditions, has enabled growers to provide 'Hass' fruit almost year-round (Griswold 1945). This has greatly facilitated the marketing of a uniform product. Year-round production of West Indian and West Indian  $\times$  Guatemalan fruit in the tropics is dependent on several cultivars, each having different maturity dates (Crane et al. 1996), and there is no market standard for tropical avocados.

Fruit diseases are serious problems in the humid tropics and subtropics. 'Fuerte' and Mexican avocados are susceptible to anthracnose or black spot caused by *Colletotrichum gloeosporioides* Penz. (Ruehle 1963). 'Collinson', 'Fuchsia' and 'Pollock' appear to be fairly resistant to *Cercospora* spot or blotch caused by *Pseudocercospora purpurea* Cooke. 'Booth 1', 'Fuchsia', 'Pollock' and 'Waldin' are moderately resistant to avocado scab caused by *Sphaceloma perseae* Jenkins.

Avocado trees grow rapidly and have a large canopy. Tree vigour and fruitfulness are inversely related (Lahav and Lavi 2002). Tree size control is currently effected by heavy pruning of trees at 4- to 5-year intervals. Compactness and either dwarf or semi-dwarf trees would be ideal for ease of harvesting, grove management and high density plantings. Currently, there are no scion selections that are appropriate.

### 3 Molecular Genetics

Molecular studies with avocado have involved the identification and application of various markers and identification of genes and ESTs.

### 3.1 Gene Cloning

The first gene cloned in avocado (pAV5) coded for cellulase (Christoffersen et al. 1984), an enzyme regulated at the transcriptional level. Tucker et al. (1987) screened a cDNA library prepared from ripe 'Hass' fruit with pAV5 and isolated the related clone pAV363. In both cases, the product was identified as cellulase because it could digest the insoluble, synthetic substrate carboxymethyl cellulose, but to date it has not been shown that either of these products can digest purified cellulose. Cass et al. (1990) screened an avocado genomic library with the cellulase cDNA probe and identified *cel1* and *cel2*; *cel1* is homologous with cellulase cDNA and represents the ripening-related cellulase gene, whereas *cel2* is divergent in its 5' end and does not resemble cellulase cDNA from ripe fruit. The *cel1* gene is responsible for the major portion of the cellulase transcripts in ripe fruit. Only *cel1* transcripts have been detected in ripe mesocarp and its accumulation has been detected in the fruit abscission zone (Tonutti et al. 1995). *Cell* expression in the mesocarp during fruit ripening is associated with abscission of mature avocado fruits.

O'Keefe et al. (1992) isolated cytochrome P-450 from the mesocarp of ripe avocado fruit, and identified it as p-chloro-N-methylaniline demethylase. Two overlapping cDNAs that accumulate during ripening were also identified as coding for P-450 (Bozak et al. 1990, 1992). The gene was designated as CYP71A1. The CYP71A1 gene product accumulates during ripening and correlates with increased total P-450 and enzyme activities, although its function during ripening is uncertain. Christoffersen et al. (1995) demonstrated that mono-terpenes, e.g., nerol and geraniol, are either hydroxylated or epoxylated by CYP71A1, although these have not been detected in ripening fruit.

Avocado polygalacturonase (PG) cDNA has been isolated from a cDNA library by heterologous hybridization with a tomato cDNA probe. The mRNA is 1900 bp and is similar in size to the reported avocado PG protein (Kanelis et al. 1991). Avocado PG is similar to tomato and corn PG and contains the conserved octa-peptide which is characteristic of PGs from plants, fungi and bacteria (Dopico et al. 1993; Kutsunai et al. 1993). Two isoforms of an endo-PG from cell walls of avocado mesocarp were isolated by Wakabayashi and Huber (2001), and had limited ability to solubilize polyuronides of fruit cell walls.

AVOe3 mRNA is ripening-related and is detected after 12 h of propylene treatment. It encodes a soluble, monomer polypeptide of 34 kDa that might be involved in ethylene biosynthesis (McGarvey et al. 1990). In another study, 23 cDNA clones were identified by differential screening of a library constructed from 7 °C-stored fruit (Dopico et al. 1993). These clones were grouped into ten families, of which six had increased expression during cold storage and normal ripening. These sequences had homologies to PG, endochitinase, a cysteine proteinase inhibitor and several stress-related proteins.

### 3.2 Genetic Markers

Molecular tools are being exploited in avocado breeding and genetic analysis for marker identification and mapping. Early studies identified isozymes as genetic markers (Torres and Bergh 1980), but recent work has involved various DNA markers, e.g., random amplified polymorphic DNA (RAPD) (Bufler and Ben-Ya'acov 1992), restriction fragment length polymorphism (RFLP) (Furnier et al. 1990; Bufler and Ben-Ya'acov 1992) and variable number of tandem repeats (VNTR), including minisatellites (Lavi et al. 1991) and microsatellites, or simple sequence repeats (SSRs) (Sharon et al. 1997).

Lavi et al. (1994) reported that the level of heterozygosity in avocado appeared to be 100% in five crosses and 90–94% in an analysis of self-pollinated progeny using mini- and microsatellites. Infrequent self-pollination would explain the high level of heterozygosity. An average heterozygosity of 0.58 was obtained by typing 59 loci with microsatellites in five avocado cultivars, and gene diversity was 0.42–0.66. A heterozygosity level of 0.79, an average of 6.1 alleles per locus and an average gene diversity of 0.78 was determined from an analysis of 11 cultivars with 17 microsatellites. However, more recently, Ashworth and Clegg (2003), employing 25 microsatellite markers, found that the average heterozygosity in 35 avocado cultivars and two wild relatives was 60.7%, ranging from 32% in *P. steyermarkii* to 84% in Fuerte and Bacon. In a subset of 15 cultivars, heterozygosity averaged 63.5% for microsatellites, compared to 41.8% for RFLPs.

The paternal origins of avocado cultivars and the frequency of outcrossing in populations of avocados have been studied using RFLP and microsatellite markers (Davis et al. 1998). Using RFLP markers, Furnier et al. (1990) determined that *P. nubigena* and *P. steyermarkii* are ancestral to *P. americana* var. *guatemalensis*. They suggested that *P. americana* consists of *P. schiedeana* and a large taxon containing other species and races. *Persea floccosa* may be a variety of *P. americana*, and the root rot-tolerant rootstock G755A probably resulted from a cross between *P. americana* and *P. schiedeana*. Using ribosomal DNA probes, Bufler and Ben-Ya'acov (1992) identified var. *drymifolia*, while var. *guatemalensis* and var. *americana* could not be distinguished. This agrees with Kopp (1966), but differs from Scora and Bergh (1990), who suggested that the three races are equally distinct from each other. Bufler and Ben-Ya'acov (1992) also used RAPDs to identify each of the avocado races and various avocado accessions.

Mhameed et al. (1997) explored the relationship among avocado cultivars and among *Persea* species using mini- and microsatellite markers. Guatemalan and West Indian races were more closely related to each other than to the Mexican race. Mhameed et al. (1997) observed that the *P. americana* races and three accessions of *P. schiedeana* were quite distinct from each other. Morphological data and DNA markers complemented each other in phylogeny studies. Mhameed et al. (1997) considered that the high levels of genetic variation in

selfing progeny challenge some assumptions about the validity of races and species.

Sharon et al. (1997) analyzed the progeny of 'Ettinger' × 'Pinkerton' using 93 microsatellites, 17 RAPD markers and 23 minisatellite markers, and developed a preliminary genetic map with 12 linkage groups having two to five markers in each group (a total of 35 markers) and covering about 357 cM. Mhameed et al. (1995) identified DFP fragments associated with skin colour, harvest duration, skin thickness, skin surface, fruit weight, seed size and peeling ability. Sharon et al. (1998) used microsatellites to identify specific bands that are associated with genes for skin gloss and seed size. The SSR marker AVAO4 (mapped to linkage group 3) is linked to a gene controlling the amount of fibre in the flesh ( $p = 0.00001$ ). Correlations between morphological and DNA markers together with a linkage map should eventually enable marker assisted selection for avocado improvement.

Ashworth and Clegg (2003), employing 25 microsatellites, uniquely differentiated 35 avocado cultivars and two wild relatives. A neighbor-joining tree, according to average shared allele distances, consisted of three clusters likely corresponding to the botanical races of avocado and intermediate clusters uniting genotypes of presumably racially hybrid origin. Considerable diversity was found particularly among accessions from Guatemala, including G810 (West Indian race), G6 (Mexican race), G755A (hybrid Guatemalan × *P. schiedeana*) and G875 (probably not *P. americana*). Low bootstrap support, even upon exclusion of (known) hybrid genotypes from the data matrix, suggested the existence of ancient hybridization, or that the botanical races originated more recently than previously thought.

## 4 Micropropagation

Clonal rootstocks with tolerance of soil-borne diseases (*Phytophthora cinnamomi* and *Rosellinia necatrix*) and of saline or calcareous soil limestone conditions are the primary goals of micropropagation. Most studies have involved shoot tips and nodal cultures of embryonic or juvenile origin (Cooper 1987; Schall 1987; Barceló-Muñoz et al. 1990; Barringer et al. 1996; Capote et al. 2000).

### 4.1 Juvenile Phase

Cooper (1987) utilized woody plant medium (WPM) formulation (Lloyd and McCown 1981) with 4.44  $\mu\text{M}$  benzyladenine (BA) for shoot proliferation and 1.3  $\mu\text{M}$  BA and 0.5  $\mu\text{M}$  indole-3-butyric acid (IBA) for shoot elongation. Barceló-Muñoz et al. (1990) and Witjaksono et al. (1999b) both described media that contained an altered  $\text{NO}_3^-$  to  $\text{NH}_4^+$  ratio, i.e., 45 mM total N content with a 4:1

$\text{NO}_3^-/\text{NH}_4^+$  ratio for the former and 40 mM total N content with 3:1  $\text{NO}_3^-$  and  $\text{NH}_4^+$  for the latter; both media were supplemented with BA.

De la Viña et al. (2001) demonstrated that shoot length and proliferation were greater on double phase medium than on semi-solid medium. Double phase medium consisted of semi-solid medium supplemented with  $2.89\ \mu\text{M}$  BA with a 3-ml of liquid medium overlay supplemented with  $0.44\ \mu\text{M}$  BA. Hyperhydricity was greater amongst shoots that developed under double phase conditions, but apical necrosis was common amongst shoots on semi-solid medium. High irradiance ( $60\text{--}85\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ) suppresses chlorophyll a and carotenoid concentrations in shoots compared to lower irradiance levels ( $35\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ ).

Rooting was facilitated if shoots were  $>1.5\ \text{cm}$  with several leaves. Less rooting occurred with shoots that developed under double phase conditions. Pliego-Alfaro (1988) obtained 60% rooting without basal medium, 100% with 0.3X MS salts (Murashige and Skoog 1962) and 10% with full-strength MS medium. Rooting can occur in the absence of sucrose (Pliego-Alfaro 1988). Callus production increased with sucrose concentration, although 3–6% sucrose is optimum for rooting. Premkumar et al. (2003) associated decreased rooting with yellowing of the leaves at 5% sucrose.

Cooper (1987) pulsed shoots for 1 s in  $1,000\text{--}3,000\ \text{mg l}^{-1}$  IBA or naphthaleneacetic acid (NAA), before transfer to basal medium. Although IBA results in slightly improved rooting, the response to NAA was more rapid. A brief pulse (3 days) with  $123\text{--}492\ \mu\text{M}$  IBA significantly increased rooting compared to continuous exposure to auxin (Pliego-Alfaro 1988); Barceló-Muñoz et al. (1990) demonstrated that lower concentrations of IBA ( $4.92\ \mu\text{M}$ ) are equally effective. De la Viña (1996) observed increased rooting following a 3-day pulse with auxin in the dark. Schall (1987) recommended continuous exposure to  $24.6\ \mu\text{M}$  IBA, and increased the rooting frequency by supplementing the medium with activated charcoal. However, Pliego-Alfaro (1988) used activated charcoal only for root development due to the inhibition of root elongation by auxin.

Endogenous auxin is essential for rooting (García-Gómez et al. 1994). Rhizogenesis does not occur in the presence of 2,3,5-triiodobenzoic acid (TIBA), although induction and development of cambial cells is not inhibited. García-Gómez et al. (1994) suggested that auxin is necessary at the early stages of rooting. Increased peroxidase activity occurs between days 3 and 6 during rooting (García-Gómez et al. 1995), and there is a close association between peroxidase activity and cambial cell division and differentiation. One hundred percent ex vitro rooting has been demonstrated following a 1-s pulse with  $3,000\ \text{mg l}^{-1}$  NAA (Cooper 1987).

## 4.2 Mature Phase

Schall (1987) initiated limited 'Fuerte' axillary bud proliferation on semi-solid, half-strength MS medium with  $22.2\ \mu\text{M}$  BA, with subsequent shoot development occurring on semi-solid medium with  $4.44\ \mu\text{M}$  BA. Harty (1985) and

Cooper (1987) reported similar success with 'Duke 7' using different semi-solid media. Harty (1985) used DF medium (Dixon and Fuller 1976) supplemented with 46  $\mu\text{M}$  kinetin, whereas Cooper (1987) used WPM with 0.44  $\mu\text{M}$  BA. Capote et al. (2000) obtained limited bud sprouting and shoot growth from etiolated axillary buds of 'Duke' on DF medium with 8.88  $\mu\text{M}$  BA and 5.77  $\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ).

Pliego-Alfaro et al. (1987) obtained proliferating shoots from nodal explants from severely pruned IV-8 rootstocks on semi-solid, half-strength MS medium with 1.3  $\mu\text{M}$  BA. Proliferating shoots were maintained on double phase medium with 2.8  $\mu\text{M}$  BA (semi-solid) and 0.4  $\mu\text{M}$  BA (liquid). This procedure has been used to multiply adult 'Duke', 'Fuerte', 'Hass' and 'Topa-Topa' (Zirari and Lionakis 1994). Hyperhydricity increases with subculture and can be controlled by culturing shoots for 2 weeks in liquid B5 medium (Gamborg et al. 1968) containing 1.3  $\mu\text{M}$  BA, followed by 6 weeks in double phase B5 medium with 2.8  $\mu\text{M}$  BA (semi solid) and 0.4  $\mu\text{M}$  BA (liquid) (Barceló-Muñoz et al. 1999). Shoots of 'Gvar-am 13' have been maintained as proliferating cultures for >2 years in  $\text{N}_{45}\text{K}$  macroelement formulation with 4.44  $\mu\text{M}$  BA, although apical necrosis must be controlled by weekly subculture (Pliego-Alfaro et al. 1987).

Micrografting of axillary buds onto in vitro germinated seedlings (Pliego-Alfaro and Murashige 1987; Barceló-Muñoz 1995) can be used to precondition shoots of cultivars that are difficult to establish. Lateral buds have been micrografted onto decapitated shoots of in vitro germinated 4-week-old seedling rootstocks with approximately 70% success. Following separation from the rootstock, shoots showed a decreased growth rate (Barceló-Muñoz 1995).

Adult phase shoots do not root easily (Pliego-Alfaro et al. 1987) and die following transfer to rooting medium (Pliego-Alfaro and Murashige 1988). Micrografting can improve rooting, e.g., 50% rooting of 'Duke-7' shoots after one graft (Pliego-Alfaro and Murashige 1988). Successive micrografts result in 90% rooting of 'Gvar-am 13' shoots (Barceló-Muñoz 1995), although proliferation is poor.

Pliego-Alfaro et al. (1987) reported 30% rooting with shoots derived from severely pruned IV-8 plants on semi-solid medium, although this could be increased to 72% if root induction occurred in liquid medium followed by semi-solid medium for development. Barceló-Muñoz et al. (1999) obtained 90% rooting with B5 medium.

### 4.3 Acclimatization

De la Viña et al. (1999) and Witjaksono et al. (1999b) investigated the relationship between photosynthetic rate,  $\text{CO}_2$  levels and plant development in vitro. De la Viña et al. (1999) observed that ribulose 1,5 biphosphate carboxylase-oxygenase (Rubisco) is low in leaves of rooted avocado shoots at high sucrose



concentrations, particularly in the presence of high CO<sub>2</sub> (1,000 μmol mol<sup>-1</sup>). Avocado plants growing in a CO<sub>2</sub>-enriched environment irrespective of the sucrose concentration showed reduced CO<sub>2</sub> assimilation. Witjaksono et al. (1999b) observed that mixotrophic plants grew better than those cultured under atmospheric CO<sub>2</sub> conditions, whereas de la Viña et al. (1999) observed that survival after transplanting was less for plants grown in low sucrose/high CO<sub>2</sub> conditions, despite their high leaf area and leaf fresh weight/stem+root fresh weight ratio. Premkumar et al. (2002, 2003) measured the effect of sucrose on leaf physiology in the culture medium, prior to acclimatizing juvenile avocado plantlets, and observed that sucrose increased utilization efficiency and starch synthesis *ex vitro* (Premkumar et al. 2003). Rooted juvenile shoots acclimatized readily under high relative humidity (Cooper 1987; Schall 1987; Barceló-Muñoz et al. 1990). Growth and development was improved after inoculation with vesicular-arbuscular mycorrhizal fungi, e.g., *Glomus fasciculatum* (Vidal et al. 1992) and *Glomus deserticola* (de la Viña et al. 1996).

Barceló-Muñoz et al. (1999) obtained 70% survival of IV-8 plants after >4 weeks in polyethylene tunnels at 100% RH, 110–120 μmol m<sup>-2</sup> s<sup>-1</sup> and 15–30 °C. Plants were exposed to increasing periods of ambient environmental conditions for 4 weeks, before transfer to open tunnels.

## 5 Micrografting for Elimination of Pathogens

Micrografting has been utilized for establishing shoot cultures of difficult cultivars and for increasing the rooting potential of mature phase shoots. Sunblotch disease of avocado is caused by avocado sunblotch viroid (ASBVd), a member of the Avsunviroidae family of viroids. ASBVd is transmitted via seeds, vegetative material, pollen and contaminated tools (Parker and Horne 1932; Wallace and Drake 1962; Desjardins et al. 1979, 1987), and eradication of affected plants is the only effective control measure.

Suarez et al. (2005) micrografted small shoot tips, consisting of the meristem with two to three leaf primordia, from *in vitro* germinated avocado seedlings of ASBVd-infected cultivars onto decapitated seedlings of two ASBVd-free cultivars. Regenerated plants were indexed for ASBVd infection by RT-PCR, which indicated that ASBVd was present in micrografts from infected scion donors, while ASBVd was not detected in micrografts from plants that tested negative. Suarez (2003) also screened embryogenic cultures and plants derived from nucellar cultures derived from infected plants, and demonstrated with RT-PCR that ASBVd was persistent. Neither *in vitro* micrografting nor nucellar culture is effective for eliminating ASBVd (Suarez 2003).



## 6 Somatic Embryogenesis

The prerequisite for applying somatic cell genetic approaches to avocado is a highly efficient regeneration protocol from single cells of elite selections. Although there were several early reports of callus initiation from various avocado tissues, the calluses were non-morphogenic (Schroeder 1956, 1961, 1971, 1980; Blumenfeld and Gazit 1971).

Pliego-Alfaro (1981) and Pliego-Alfaro and Murashige (1988) induced embryogenic cultures from avocado zygotic embryos of openly pollinated 'Hass' (Mexican  $\times$  Guatemalan). These results were verified with openly pollinated 'Fuerte' (Mexican  $\times$  Guatemalan) and 'Duke 7' (Mexican) by Mooney and van Staden (1987), Raviv et al. (1998) and Witjaksono and Litz (1999a, b). Zygotic embryos representing different developmental stages have been utilized as explants. Mooney and van Staden (1987) utilized 0.1- to 0.5-mm embryos from 3- to 4-mm fruits, Pliego-Alfaro and Murashige (1988) used 0.6- to 0.8-mm embryos from 9-mm fruits and Raviv et al. (1998) used 7- to 10-mm embryos. Surface-sterilized immature fruit were bisected longitudinally, and the zygotic embryo placed in contact with medium. Embryogenic cultures can also be induced from the nucellus of immature avocado seeds (Witjaksono et al. 1999a).

Induction medium has generally been semi-solid MS medium supplemented with  $0.1 \text{ mg l}^{-1}$  thiamine HCl,  $100 \text{ mg l}^{-1}$  myo-inositol,  $30 \text{ g l}^{-1}$  sucrose and  $0.41 \text{ }\mu\text{M}$  picloram. Witjaksono and Litz (1999a) observed that an induction basal medium consisting of B5 major salts with MS minor salts and organic components is superior. Embryogenic cultures consisted of proembryonic masses (PEMs) and hyperhydric somatic embryos. Cultures were maintained in darkness at  $25^\circ\text{C}$ .

Embryogenic cultures proliferate rapidly on semi-solid MS or modified B5 induction media. Embryogenic suspension cultures, however, are easily established and are optimum for proliferation (Witjaksono and Litz 1999a). Modified MS medium containing  $12 \text{ mg l}^{-1}$   $\text{NH}_4\text{NO}_3$  and  $30.3 \text{ mg l}^{-1}$   $\text{KNO}_3$  results in the highest fresh weight increase compared to other media (Witjaksono and Litz 1999b), with biweekly subcultures. Inocula consist of 0.5 or 1 g embryogenic culture in 40 or 80 ml liquid maintenance medium in 125- or 250-ml Erlenmeyer flasks, respectively. Optimizing medium-term maintenance of embryogenic cultures is essential for many types of in vitro manipulation.

Two distinct phenotypes of embryogenic avocado cultures have been recognized (Witjaksono and Litz 1999a), and these responses are genotype-dependent: (1) the PEM-phenotype, which describes the few genotypes that proliferate as PEMs without differentiation of cotyledons in the presence of auxin, and (2) the SE-phenotype, which describes the response of most genotypes that differentiate heart and later developmental stages of somatic embryos in the presence of auxin. Suspension cultures of the SE-phenotype must be sieved at each subculture, with only the  $<0.8\text{-mm}$  fraction being inoculated into new maintenance medium. Under maintenance conditions, the loss of

embryogenic potential is cultivar-dependent (Witjaksono and Litz 1999a) and varies from 3 months to >2 years for 'Yon' and 'Esther', respectively. Cultures on semi-solid maintenance medium are incubated in darkness at 25 °C, and suspension cultures are incubated in semi-darkness.

Mooney and Van Staden (1987) and Pliego-Alfaro and Murashige (1988) reported that somatic embryo development followed the transfer of embryogenic cultures to semi-solid medium without picloram. However, the absence of auxin was not essential for somatic embryo development of SE-phenotype cultures, which defined the response of most genotypes (Witjaksono and Litz 1999a). Raviv et al. (1998) also observed that cotyledonary somatic embryos can develop on semi-solid medium supplemented with auxin. Somatic embryo development following transfer from maintenance to maturation media is genotype-dependent; development from SE-phenotype cultures is more efficient (Witjaksono and Litz 1999a, b).

The optimum conditions for somatic embryo maturation include MS medium, which has been supplemented with 30 g l<sup>-1</sup> sucrose, 4 mg l<sup>-1</sup> thiamine HCl, 100 mg l<sup>-1</sup> myo-inositol and 6.0 g l<sup>-1</sup> gellan gum in the dark at 25 °C (Witjaksono and Litz 1999b). Hyperhydricity of somatic embryos can be reversed by transfer onto medium with higher gellan gum and sucrose concentrations. Plants are recovered from fully enlarged, opaque mature somatic embryos (0.8–1.0 cm diameter) on semi solid germination medium, i.e., MS medium supplemented with 4.44 M BA and 2.89 µM GA<sub>3</sub> (Witjaksono and Litz 1999b). Most somatic embryos are abnormal due to failure of the apical meristem to become organized (Pliego-Alfaro and Murashige 1988). MS medium containing 6 g l<sup>-1</sup> gellan gum, 45 mg l<sup>-1</sup> sucrose and 20% (v/v) coconut water has been used routinely for somatic embryo development (Efendi 2003; Witjaksono and Litz 2004). Filter-sterilized coconut water can increase the frequency of recovery of somatic embryos with apical meristems (Witjaksono and Litz 2002). Somatic embryo shoots can be rescued by micropropagation (Witjaksono et al. 1999b), followed by root induction by pulsing them for 3 days on MS medium supplemented with 122.6 µM IBA (Pliego-Alfaro 1988). Alternatively, somatic embryo-derived shoots can be micrografted onto decapitated in vitro-germinated seedlings (Raharjo and Litz 2005).

## 7 Protoplast Isolation and Culture

Early reports of avocado protoplast isolation and culture involved non-morphogenic cultures for studies of avocado sunblotch viroid (Blickel et al. 1986) and fruit ripening (Percival et al. 1991). Protoplasts were also isolated from non-morphogenic suspensions of *P. cinerascens* and *P. pachypoda* in the subgenus *Eriodaphne* (Witjaksono and Litz 2000). The isolation and culture of protoplasts has been described from embryogenic avocado cultures and plant regeneration (Witjaksono et al. 1998). The isolation and culture of protoplasts

and their regeneration is more efficient with PEM-phenotypes than with SE-phenotype cultures. Medium osmolarity, nitrogen source and plating density all affect the development of PEMs from protoplasts in liquid medium. The optimum conditions for recovery of PEMs from protoplasts are 0.4 M MS<sup>-</sup>8P liquid medium,  $0.8 \times 10^5$  ml<sup>-1</sup> protoplast density and a plating efficiency after 12 days of approx. 25%. Protoplast-derived PEMs can develop on maturation medium, and plants have been regenerated from somatic embryos at low frequency.

Resistance to PRR, which has been identified in species within the subgenus *Eriodaphne*, is not possible in avocado due to graft and sexual incompatibility barriers between *Persea* spp. within this subgenus and species in the subgenus *Persea* (Frohlich et al. 1958). Somatic hybridization was proposed as an approach to achieve hybridization between species in the two subgenera (Pliego-Alfaro and Bergh 1992). Witjaksono (1997) attempted somatic hybridization of avocado with PRR-resistant species by fusion between protoplasts from embryogenic avocado cultures and leaf or callus protoplasts of PRR-resistant species. However, the results of this study were inconclusive. Somatic hybrids were recovered as a result of the fusion of embryogenic avocado protoplasts with non-morphogenic callus protoplasts of *Persea* spp. in the subgenus *Eriodaphne* (Witjaksono and Litz, unpublished data), but the somatic embryos lacked an apical meristem, and plants could not be recovered.

## 8 In Vitro Mutation Induction

Avocado trees have been selected with somatic mutations that affect tree architecture, leaf shape, size and colour, and fruit shape, size and skin texture. Off types of 'Fuerte', including 'Weisel', 'Newman' and 'de Bard', have been selected (Hodgson 1945). The rootstock 'D9', which originated from irradiated 'Duke', has good resistance to PRR. Scions grafted on 'D9' show a dwarfing effect of the rootstock, and are more productive than on 'Martin Grande', but less so on 'Borchard' and 'Duke 7' (Arpaia et al. 1992). 'Hass' irradiated with 13 Gy showed reduced vegetative growth, and greater flowering and fruit set (de la Cruz-Torres et al. 1995a). Irradiation at 15 Gy resulted in variability in height, rootstock and graft diameter, stomatal density and internode length (de la Cruz-Torres et al. 1995b). There was no apparent separation of chimeras in these studies.

The radiation sensitivity of embryogenic cultures of 'Fuerte' and 'T362' was described by Witjaksono and Litz (2004), together with the effects of gamma irradiation on somatic embryo development from irradiated cultures. Two weeks after irradiation of 'Fuerte' and 4 weeks after irradiation of 'T362', the approximate PD<sub>50</sub> was 35 Gy. Irradiation did not significantly affect the number of early stage 'Fuerte' somatic embryos that developed from irradiated cultures. However, 10–50 Gy inhibited subsequent somatic embryo development. The

number of intermediate and mature stages of 'T362' somatic embryos that developed following irradiation of embryogenic cultures at 25–50 Gy was inhibited, while somatic embryo maturation was inhibited by 50 Gy. Witjaksono (2001) describes that the objectives of this study were to select *in vitro* for resistance to the culture filtrate of *Phytophthora cinnamomi* and to regenerate plants with resistance to PRR.

## 9 Genetic Transformation

Genetic transformation of avocado has been based upon the embryogenic pathway. Cruz-Hernandez et al. (1998) describe the basic protocol for transforming avocado. Growth of PEM-phenotype embryogenic suspensions is suppressed by 50% with 50 mg l<sup>-1</sup> kanamycin sulfate, whereas complete suppression of growth of embryogenic cultures on semi-solid medium requires 200 mg l<sup>-1</sup> kanamycin sulfate. A two-step selection procedure was described for PEM-phenotype cultures for recovery of genetic transformants. PEM-phenotype embryogenic cultures on semi-solid maintenance medium are gently abraded with a soft camel hairbrush, and then incubated with acetosyringone-activated *Agrobacterium tumefaciens* in liquid maintenance medium for 3 days at 100 rpm. Strain 9749 ASE2 was used containing a co-integrate vector pMON9749 with a selectable kanamycin-resistant marker (*nptII*) and *gus* ( $\beta$ -glucuronidase). *Agrobacterium tumefaciens* was eliminated by incubating the cultures in maintenance medium containing 50 mg l<sup>-1</sup> kanamycin sulfate and 200 mg l<sup>-1</sup> cefotaxime. An initial selection for antibiotic resistance in liquid maintenance medium containing 50 mg l<sup>-1</sup> kanamycin sulfate for 2–4 months was followed by a second, more intense selection in the presence of 100 mg l<sup>-1</sup> kanamycin sulfate for 2 months. Finally, the cultures were cultured in 200 mg l<sup>-1</sup> kanamycin sulfate to eliminate chimaeras. Somatic embryo development was initiated on maturation medium (lacking kanamycin sulfate), followed by sub-culture on maturation medium containing kanamycin sulfate. Transformed somatic embryos stained positively for *gus* (Jefferson 1987), and the integration of *nptII* and *gus* genes into the avocado genome was confirmed by PCR and Southern hybridization (Miller 1972; Doyle and Doyle 1990). However, transgenic plants were not regenerated.

Genetic transformation could be used to address some important rootstock and scion breeding objectives of avocado. The primary breeding objectives have been to develop improved avocado rootstocks with greater resistance to PRR and scion cultivars with resistance to foliar and fruit diseases, using genes that encode for disease resistance. Controlling fruit ripening is an important breeding objective that would have two important ramifications, namely (1) to permit on-tree storage of West Indian and West Indian  $\times$  Guatemalan avocados, and (2) to extend the post-harvest storage time of all types of avocados.

Embryogenic avocado cultures have been transformed with genes which could affect different horticultural traits, and plants have been regenerated (Raharjo et al. 2003). The initial glasshouse trials commenced in 2003. Avocado has been transformed with the gene for S-adenosylmethionine (SAM) hydrolase (SAMase) in order to control fruit ripening. SAM hydrolase blocks ethylene production, by converting SAM to a non-toxic by-product that cannot be converted to ACC (Good et al. 1994). The SAMase gene is in pAG4092 under the control of an avocado fruit-specific cellulase promoter with *nptII* as a selectable marker. Efendi (2003) described the recovery of transformed somatic embryos that contain the SAMase gene, and Raharjo and Litz (2003) rescued shoots from transformed somatic embryos by micrografting them on decapitated in vitro-germinated seedlings.

Embryogenic avocado cultures have also been genetically transformed with the PR-related genes,  $\beta$ -1,6-glucanase, chitinase and the antifungal protein (AFP) gene in order to address the problem of PRR of avocado rootstocks (Raharjo, Witjaksono, Gomez-Lim and Litz, unpublished data). The AFP gene was in pGPTV-AFP together with *uidA*, the gene for resistance to phosphinotricin (PPT) and the CaMV 35S promoter. Glucanase and chitinase have been cloned in pGPTV-CG, together with *uidA*, *bar* and the CaMV 35S promoter. PPT selection has been used for both AFP and CG. Although transformed somatic embryo-derived shoots are rescued routinely by micrografting, this strategy has no utility for rootstocks. Therefore, transformed shoots that have been rescued by micrografting are air layered ex vitro in order to produce transformed rootstocks (Raharjo and Litz, unpublished data).

## 10 Cryopreservation

High costs in terms of land, labour, pests, diseases and extreme weather have limited the conservation of avocado genetic resources ex situ. Moreover, genetic diversity within the genus *Persea* is large, and collections of species, subspecies and cultivars for breeding and genetic studies must therefore also be large. Genetic manipulation of avocado at the cell level is dependent on a continuous supply of embryogenic material. Witjaksono and Litz (1999a) reported that embryogenic competence of certain genotypes is lost soon after induction. Therefore, the annual replacement of important embryogenic lines has been essential. Cryopreservation is useful for backing up existing collections, and thereby could impact long-term maintenance of clonal *Persea* genetic resources, and ensure a continuous supply of embryogenic cultures.

Efendi (2003) and Efendi and Litz (2003) successfully cryopreserved embryogenic avocado cultures. Somatic embryos were recovered and plants were regenerated using the two procedures of (1) slow cooling ( $-1^{\circ}\text{C min}^{-1}$  from room temperature down to  $-80^{\circ}\text{C}$ , followed by rapid cooling to  $-196^{\circ}\text{C}$ ), and

(2) vitrification, i.e., rapid cooling from room temperature to  $-196^{\circ}\text{C}$ . For slow cooling, embryogenic cultures were cryoprotected with dimethylsulfoxide and glycerol and cooled at  $-1^{\circ}\text{C min}^{-1}$  to  $-80^{\circ}\text{C}$  in “Mr. Frosty” containers (Nalge Nunc, Rochester, New York, USA). The cryovials were then plunged into liquid nitrogen. For vitrification, embryogenic cultures were suspended in PVS2 vitrification solution (Sakai et al. 1991), consisting of glycerol (30%), ethylene glycol (15%) and DMSO (15%) for 15 min, prior to rapid cooling in liquid nitrogen. The cryovials were removed from liquid nitrogen, warmed rapidly to room temperature, washed thoroughly with maintenance medium and plated on semi-solid maintenance medium. The procedures for somatic embryo development and plant recovery followed the protocols described earlier.

## 11 Conclusions

Different biotechnological approaches can be utilized to address important breeding objectives of avocado. Shoot tip and nodal culture and somatic embryogenesis have potential as alternative vegetative propagation procedures for cloning PRR-resistant rootstocks. It is likely that more avocado genes will be identified by the tools of genomics research, and homologies will be established with genes from model species, e.g., *Arabidopsis*. An ongoing project at CINVESTAV involves the construction of a subtractive library from root tissue of a resistant cultivar infected by *Phytophthora* at the earliest stages, and cloning and identifying those avocado genes responding primarily to the infection. Identification of genes coding for important traits, including yield, fruit size and shape, disease and pest resistance, will improve classical breeding by marker assisted selection and provide suitable genes for generation of transgenic plants with desired characteristics. Cryopreservation of avocado is sure to be an important tool for the management of genetic resources, and to ensure a supply of morphogenically competent cells for somatic cell genetic studies.

**Acknowledgements.** The assistance of the California Avocado Commission is gratefully acknowledged. This work has been published in the Florida Agricultural Experiment Station Journal Series no. R-10753.

## References

- Adato I, Gazit S (1977) Postharvest response of avocado fruits of different maturity to delayed ethylene treatments. *Plant Physiol* 53:899–902
- Arpaia ML, Bender GS, Witney GW (1992) Avocado clonal rootstock production trial. In: Lovatt C, Holthe PA, Arpaia ML (eds) *Proceedings of the Second World Avocado Congress*, vol. 1. University of California, Riverside, pp 305–310
- Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. *Plant Mol Biol Rep* 9:208–218



- Ashworth VE, Clegg MT (2003) Microsatellite markers in avocado (*Persea americana* Mill.): genealogical relationships among cultivated avocado genotypes. *J Hered* 94:407–415
- Barceló-Muñoz A (1995) Micropropagación de aguacate (*Persea americana* Mill.). PhD Thesis, Facultad de Ciencias, Universidad de Málaga
- Barceló-Muñoz A, Encina CL, Simón-Pérez E, Pliego-Alfaro F (1999) Micropropagation of adult avocado. *Plant Cell Tissue Organ Cult* 58:11–17
- Barceló-Muñoz A, Pliego-Alfaro F, Barea JM (1990) Micropropagación de aguacate (*Persea americana* Mill.) en fase juvenil. *Actas Hort* 1:503–506
- Barringer SA, Mohamed-Yassen Y, Splittstoesser WE (1996) In vitro multiplication and plantlet establishment of avocado. *In Vitro Cell Dev Biol-Plant* 32:119–1221
- Ben-Ya'acov A (1972) Avocado rootstock–scion relationship: a long-term, large-scale field research project. *Calif Avocado Soc Yearbook* 55:158–161
- Ben-Ya'acov A, Michelson E (1995) Avocado rootstocks. *Hort Rev* 17:381–429
- Ben-Ya'acov A, Michelson E, Sela I (1974) Avocado rootstock–scion relationships: a long-term, large-scale field research project. Sensitivity of avocado rootstocks to inadequate soil aeration. *Calif Avocado Soc Yearbook* 57:108–113
- Bergh BO (1957) Avocado breeding in California. *Proc Fla State Hortic Soc* 70:284–290
- Bergh BO, Ellstrand NC (1986) Taxonomy of the avocado. *Calif Avocado Soc Yearbook* 70:135–145
- Bergh BO, Lahav E (1996) Avocados. In: Janick J, Moore JN (eds) *Fruit breeding*, vol. I. Tree and tropical fruits. John Wiley, West Lafayette, pp 113–166
- Blickel W, Muhlbach HP, Sanger HL (1986) Conditions for the isolation of protoplasts from callus cultures of avocado (*Persea americana*). *Proc 6th Int Congr on Plant Cell and Tissue Culture*, University of Minnesota, Minneapolis, p 357
- Blumenfeld A, Gazit S (1971) Growth of avocado fruit callus and its relation to exogenous and endogenous cytokinin. *Physiol Plant* 25:369–371
- Bozak KR, Yu H, Sirevag R, Christoffersen RE (1990) Cloning and sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. *Proc Natl Acad Sci USA* 87:3904–3908
- Bozak KR, O'Keefe DP, Christoffersen RE (1992) Expression of ripening-related avocado (*Persea americana*) cytochrome P450 in yeast. *Plant Physiol* 100:1976–1981
- Bufler G, Ben-Ya'acov A (1992) A study of the avocado germplasm resources, 1988–1990. Ribosomal DNA repeat unit polymorphism in avocado. In: Lovatt C, Holthe PA, Arpaia ML (eds) *Proceedings of the Second World Avocado Congress*, vol. 2. University of California, Riverside, pp 545–550
- Capote M, Rodríguez NN, Blanco M (2000) In vitro propagation of avocado. *Trop Fruits Newslett* 36/37:3–7
- Cass LG, Kirven KA, Christoffersen RE (1990) Isolation and characterization of a cellulase gene family member expressed during avocado fruit ripening. *Mol Gen Genet* 223:76–86
- Christoffersen RE, Tucker ML, Laties GG (1984) Cellulase gene expression in ripening avocado fruit: the accumulation of cellulase mRNA and protein as demonstrated by cDNA hybridization and immunodetection. *Plant Mol Biol* 3:385–391
- Christoffersen RE, Percival FW, Bozak KR (1995) Functional and DNA sequence divergence of the CYP71 gene family in higher plants. *Drug Metab Interact* 12:207–219
- Coffey MD (1987) *Phytophthora* root rot of avocado. *Plant Dis* 71:1046–1052
- Cooper PA (1987) Advances in the micropropagation of avocado (*Persea americana* Mill.). *Acta Hort* 212:571–575
- Crane JH, Balerdi CF, Campbell CW (1996) The avocado circular 1034. Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville
- Cruz-Hernandez A, Witjaksono, Litz RE, Gomez-Lim M (1998) *Agrobacterium tumefaciens*-mediated transformation of embryogenic avocado cultures and regeneration of somatic embryos. *Plant Cell Rep* 17:497–503
- Davis J, Henderson D, Kobayashi M, Clegg MT (1998) Genealogical relationships among cultivated avocado as revealed through RFLP analyses. *J Hered* 89:319–323
- de la Cruz-Torres E, Arriaga MR, Guevara CS (1995a) Advances on the radioinduced mutation breeding programme on avocado at CICTAMEX. *Proc 3rd World Avocado Congr*, Tel Aviv, p 120



- de la Cruz-Torres E, Arriaga MR, Guevara CS (1995b) Advances on the radioinduced mutation breeding programme on Hass avocado. Proc 3rd World Avocado Congr, Tel Aviv, p 148
- de la Viña G (1996) Estudio de algunos factores que afectan la capacidad de aclimatación de plantas de aguacate micropropagado. PhD Thesis, Facultad de Ciencias, Universidad de Málaga
- de la Viña G, Azcón Aguilar C, Barea JM, Barceló A, Pliego-Alfaro F (1996) Effects of arbuscular mycorrhizas in the acclimatization of micropropagated avocado plants. In: Azcón Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems: from genes to plant development. EUR Report 16728-EN. Directorate-General XII Science Research and Development, Brussels, pp 491–494
- de la Viña G, Pliego-Alfaro F, Driscoll SP, Mitchel VJ, Parry MA, Lawlor DW (1999) Effects of CO<sub>2</sub> and sugars on photosynthesis and composition of avocado leaves grown in vitro. Plant Physiol Biochem 37:587–595
- de la Viña G, Barceló-Muñoz A, Pliego-Alfaro F (2001) Effect of culture media and irradiance level on growth and morphology of *Persea americana* Mill. microcuttings. Plant Cell Tissue Organ Cult 65:229–237
- Desjardins PR, Drake RJ, Atkins EL, Bergh BO (1979) Pollen transmission of avocado sunblotch virus experimentally demonstrated. Calif Agric 33:14–15
- Desjardins PR, Saski PJ, Drake RJ (1987) Chemical inactivation of avocado sunblotch viroid on pruning and propagation tools. Calif Avocado Soc Yearbook 71:259–261
- Dixon A, Fuller KW (1976) Effect of synthetic auxin levels on *Phaseolus vulgaris* L. Physiol Plant Pathol 11:287–292
- Dopico B, Lowe AL, Wilson ID, Merodio C, Grierson D (1993) Cloning and characterization of avocado fruit mRNAs and their expression during ripening and low temperature storage. Plant Mol Biol 21:437–449
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:12–14
- Eaks IL (1980) Respiratory rate, ethylene production and ripening response of avocado fruit to ethylene or propylene following harvest maturities. J Am Soc Hortic Sci 105:744–747
- Efendi D (2003) Transformation and cryopreservation of embryogenic avocado (*Persea americana* Mill.) cultures. PhD Dissertation, University of Florida, Gainesville
- Efendi D, Litz RE (2003) Cryopreservation of avocado. Proc 5th Congr Mundial de Aguacate, vol II, Malaga, pp 111–114
- Ellstrand NC, Lee JM, Bergh BO, Coffey MD, Zentmyer GA (1986) Isozymes confirm hybrid parentage for 'G755' selections. Calif Avocado Soc Yearbook 70:199–203
- Frohlich EF, Schroeder CA, Zentmyer GA (1958) Graft compatibility in the genus *Persea*. Calif Avocado Soc Yearbook 58:102–105
- Furnier GR, Cummings MP, Clegg MT (1990) Evolution of the avocados as revealed by DNA restriction site variation. J Hered 81:183–188
- Gamborg OL, Miller RA, Ojima K (1968) Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
- Garcia AV (1975) Cytogenetic studies in the genus *Persea* (Lauraceae). I: Karyology of seven species. Can J Genet Cytol 17:173–180
- García-Gómez ML, Sánchez-Romero C, Barceló-Muñoz A, Heredia A, Pliego-Alfaro F (1994) Levels of endogenous indole-3-acetic acid and indole-3-acetyl-aspartic acid during adventitious root formation in avocado microcuttings. J Exp Bot 45:865–870
- García-Gómez ML, Sánchez-Romero C, Barceló-Muñoz A, Heredia A, Pliego-Alfaro F (1995) Peroxidase activity during adventitious root formation in avocado microcuttings. Can J Bot 73:1522–1526
- Good X, Kellogg JA, Wagoner W, Langhoff D, Matsumura W, Bestwick RK (1994) Reduced ethylene synthesis by transgenic tomatoes expressing S-adenosylmethionine hydrolase. Plant Mol Biol 26:781–790
- Griswold HB (1945) The Hass avocado. Calif Avocado Soc Yearbook 1945, pp 27–31
- Harty PA (1985) Propagation of avocado by tissue culture: development of a culture medium for multiplication of shoots. S Afr Avocado Growers' Assoc Yearbook 8:70–71

- Hodgson RW (1945) Suggestive evidence of the existence of strains of 'Fuerte' avocado variety. Calif Avocado Soc Yearbook 1945, pp 24–26
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS fusion system. Plant Mol Biol Rep 5:387–405
- Kadman A (1968) Selection of avocado rootstocks suitable for use with saline irrigation water. Calif Avocado Soc Yearbook 52:145–147
- Kadman A, Ben-Ya'acov A (1976) Selection of avocado rootstocks for saline conditions. Acta Hort 57:189–197
- Kanellis AK, Solomos T, Roubelakis-Angelakis KA (1991) Suppression of cellulase and polygalacturonase and induction of alcohol dehydrogenase isoenzymes in avocado fruit mesocarp subjected to oxygen stress. Plant Physiol 96:269–274
- Kopp L (1966) A taxonomic revision of the genus *Persea* in the western hemisphere (*Persea-Lauracea*). Mem New York Bot Garden 14:1–120
- Kostermans AJGH (1952) A historical survey of the Lauraceae. J Sci Res 1:83–95; 113–127; 141–159
- Kutsunai S, Lin AC, Percival FW, Laties GG, Christoffersen RE (1993) Ripening related polygalacturonase cDNA from avocado. Plant Physiol 103:289–290
- Lahav E, Kalmar D (1977) Water requirement of avocado in Israel. II: influence on yield, fruit growth and oil content. Aust J Agric Res 28:869–877
- Lahav E, Lavi U (2002) Genetics and breeding. In: Whitley AW, Schaffer B, Wolstenholme BN (eds) Avocado botany production and uses. CAB International, Wallingford, Oxon, pp 39–69
- Lavi U, Hillel J, Vainstein A, Lahav E, Sharon D (1991) Application of DNA fingerprints for identification and genetic analysis of avocado. J Am Soc Hortic Sci 116:1078–1081
- Lavi U, Lahav E, Degani C, Gazit S, Hillel J (1993a) Genetic variance components and heritabilities of several avocado traits. J Am Soc Hortic Sci 118:400–404
- Lavi U, Lahav E, Degani C, Gazit S (1993b) Genetics of flower color, flowering group and anise scent in avocado. J Hered 84:82–84
- Lavi U, Akkaya M, Bhagwat A, Cregan PB (1994) Methodology of generation and characterization of simple sequence repeat DNA markers in avocado (*Persea americana* M.). Euphytica 80:171–177
- Lloyd GB, McCown BH (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Int Plant Prop Soc Com Proc 30:421–427
- McGarvey DG, Sirevag R, Christoffersen RE (1990) Ripening related gene from avocado fruit. Plant Physiol 98:554–559
- McMurchie EJ, McGlasson WB, Eaks IL (1972) Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature 237:235–236
- Mhameed S, Hillel J, Lahav E, Sharon D, Lavi U (1995) Genetic association between DNA fingerprint fragment and loci controlling agriculturally important traits in avocado (*Persea americana* Mill.). Euphytica 81:81–87
- Mhameed S, Sharon D, Kaufman D, Lahav E, Hillel J, Degani C, Lavi U (1997) Genetic relationship within avocado (*Persea americana* Mill.) cultivars and between *Persea* species. Theor Appl Genet 94:279–284
- Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Mooney PA, van Staden J (1987) Induction of embryogenesis in callus from immature embryos of *Persea americana*. Can J Bot 65:622–626
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
- O'Keefe DP, Bozak KR, Christoffersen RE, Tepperman JA, Dean C, Harder PA (1992) Endogenous and engineered cytochrome P-450 mono-oxygenase in plants. Biochem Soc Trans 20:357–361
- Parker ER, Horne WT (1932) The transmission of avocado sun blotch. Calif Avocado Soc Yearbook 16:50–56
- Percival FW, Cass LG, Bozak C, Christoffersen R (1991) Avocado fruit protoplasts: a cellular model system for ripening studies. Plant Cell Rep 10:512–516

- Pliego-Alfaro F (1981) A morphogenetic study of the avocado (*Persea americana* Mill.) in vitro. PhD Diss, University of California, Riverside, California
- Pliego-Alfaro F (1988) Development of an in vitro rooting bioassay using juvenile-phase stem cuttings of *Persea americana* Mill. *J Hortic Sci* 63:295–301
- Pliego-Alfaro F, Bergh BO (1992) Avocado. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford, Oxon, pp 323–333
- Pliego-Alfaro F, Murashige T (1987) Possible rejuvenation of adult avocado by graftage onto juvenile rootstocks in vitro. *HortScience* 22:1321–1324
- Pliego-Alfaro F, Murashige T (1988) Somatic embryogenesis in avocado (*Persea americana* Mill.) in vitro. *Plant Cell Tissue Organ Cult* 12:61–66
- Pliego-Alfaro F, López-Encina C, Barceló-Muñoz A (1987) Propagation of avocado rootstocks by tissue culture. *S Afr Avocado Growers' Assoc Yearbook* 10:36–39
- Popenoe W (1941) The avocado – a horticultural problem. *Trop Agric* 18:3–7
- Premkumar A, Barceló-Muñoz A, Pliego-Alfaro F, Quesada MA, Mercado JA (2002) Influences of exogenous sucrose on juvenile avocado during in vitro cultivation and subsequent ex vitro acclimatization. *Trees* 16:569–575
- Premkumar A, Barceló-Muñoz A, Pliego-Alfaro F, Quesada MA, Mercado JA (2003) Physiological influence of sucrose concentration on in vitro rooting, growth, endogenous sugars and ex vitro survival of juvenile avocado. *J Hortic Sci Biotechnol* 78:46–50
- Raharjo S, Litz RE (2003) Rescue of genetically engineered avocado by micrografting. *Proc 5th Congr Mundial de Aguacate*, vol II, Malaga, pp 119–122
- Raharjo S, Witjaksono, Efendi D, Gomez-Lim MA, Suarez I, Litz RE (2003) Genetic transformation of avocado. *Proc 5th Congr Mundial de Aguacate*, vol II, Malaga, pp 115–118
- Raharjo SHT, Litz RE (2005) In vitro and ex vivo grafting for somatic embryo rescue and plant recovery in avocado (*Persea americana* Mill.). *Plant Cell Tissue Organ Cult* 82:1–9
- Raviv A, Avenido RA, Tisalona LF, Damasco OP, Mendoza EMT, Pinkas Y, Zilkah S (1998) Callus and somatic embryogenesis of *Persea* species. *Plant Tissue Cult Biotechnol* 4:196–206
- Ruehle GD (1963) The Florida avocado industry. *Univ Fla Agric Exp Stn Bull* 602
- Sakai A, Kobayashi S, Oyama I (1991) Cryopreservation of nucellar cells of naval range (*Citrus sinensis* Osb. var *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep* 9:30–33
- Sánchez-Colin S, Barrientos-Priego A (1987) Avocado production and breeding in Mexico. *S Afr Avocado Growers' Assoc Yearbook* 10:24–26
- Schall S (1987) La multiplication de l'avocatier (*Persea americana* Mill. cv. Fuerte) par microbouturage in vitro. *Fruits* 42:171–176
- Schroeder CA (1956) Growth of avocado fruit tissue on artificial media. *Calif Avocado Soc Yearbook* 40:165–168
- Schroeder CA (1961) Some morphological aspects of fruit tissue grown in vitro. *Bot Gaz* 112:198–204
- Schroeder CA (1971) The response of avocado pericarp tissue to temperature and light in vitro. *Calif Avocado Soc Yearbook* 54:85–89
- Schroeder CA (1980) Avocado tissue in vitro. *Calif Avocado Soc Yearbook* 64:139–141
- Scora RW, Bergh BO (1990) The origin and taxonomy of avocado (*Persea americana* Mill.) Lauraceae. *Acta Hort* 275:387–394
- Scora RW, Wolstenholme BN, Lavi U (2002) Taxonomy and botany. In: Whitley AW, Schaffer B, Wolstenholme BN (eds) *Avocado botany production and uses*. CAB International, Wallingford, Oxon, pp 15–38
- Sharon D, Cregan PB, Mhameed S, Hillel J, Lahav E, Lavi U (1997) An integrated genetic linkage map of avocado. *Theor Appl Genet* 95:911–921
- Sharon D, Hillel J, Mhameed S, Cregan TB, Lahav E, Lavi U (1998) Association between DNA markers and loci controlling avocado traits. *J Am Soc Hortic Sci* 123:1016–1022
- Suarez IE (2003) Avocado sunblotch viroid (ASBVd) in cell and tissue cultures of avocado (*Persea americana*). PhD Diss, University of Florida, Gainesville
- Suarez IE, Schnell RA, Kuhn DA, Litz RE (2005) Micrografting of ASBVd-infected avocado (*Persea americana* Mill.) plants. *Plant Cell Tissue Organ Cult* 80:179–185

- Tonutti P, Cass LG, Christoffersen RE (1995) The expression of cellulase gene family members during induced avocado fruit abscission and ripening. *Plant Cell Environ* 18:709–713
- Torres AM, Bergh BO (1980) Fruit and leaf isozymes as genetic markers in avocado. *J Am Soc Hortic Sci* 105:614–619
- Tsao PH, Alizadeh A, Krausman E (1992) Susceptibility to *Phytophthora citricola* of certain avocado rootstock cultivars known to be tolerant to *Phytophthora cinnamomi*. In: Lovatt C, Holthe PA, Arpaia ML (eds) *Proceedings of the Second World Avocado Congress*, vol. 1. University of California, Riverside, pp 89–94
- Tucker ML, Durbin ML, Clegg MT, Lewis LN (1987) Avocado cellulase: nucleotide sequence of a putative full-length cDNA clone and evidence for a small gene family. *Plant Mol Biol* 9:197–203
- Vidal MT, Azcón-Aguilar C, Barea JM, Pliego-Alfaro F (1992) Mycorrhizal inoculation enhances growth and development of micropropagated plants of avocado. *HortScience* 27:785–787
- Wakabayashi K, Huber DJ (2001) Purification and catalytic properties of polygalacturonase isoforms from ripe avocado (*Persea americana*) fruit mesocarp. *Physiol Plant* 113:210–216
- Wallace JM, Drake R.J (1962) A high rate of seed transmission of avocado sunblotch from symptomless trees and the origin of such trees. *Phytopathology* 52:237–241
- Whiley AW (1992) *Persea americana* Miller. In: Verheij EWM, Coronel RE (eds) *Plant resources of South-East Asia* no. 2 – edible fruits and nuts. Pudoc-DLO, Wageningen, pp 249–254
- Whiley AW, Schaffer B (1994) Avocado. In: Schaffer B, Andersen PC (eds) *Handbook of environmental physiology of fruit crops*, vol. 2. Subtropical and tropical crops. CRC Press, Boca Raton, Florida, pp 165–197
- Witjaksono (1997) Development of protocols for avocado tissue culture: somatic embryogenesis, protoplast culture, shoot proliferation and protoplast fusion. PhD Diss, University of Florida, Gainesville
- Witjaksono (2001) Avocado rootstock improvement through induced mutation and biotechnology. Improvement of tropical and subtropical fruit trees through induced mutations and biotechnology. Report of the First Research Co-ordination Meeting of FAO/IAEA Co-ordinated Research Project, Vienna, pp 39–42
- Witjaksono, Litz RE (1999a) Induction and growth characteristics of embryogenic avocado (*Persea americana* Mill.) cultures. *Plant Cell Tissue Organ Cult* 58:19–29
- Witjaksono, Litz RE (1999b) Maturation and germination of avocado (*Persea americana* Mill.) somatic embryos. *Plant Cell Tissue Organ Cult* 58:141–148
- Witjaksono, Litz RE (2000) Cell suspension culture of *Persea pachypoda* and *P. cinerascens*. In *Vitro Cell Dev Biol* 36:69A
- Witjaksono, Litz RE (2002) Somatic embryogenesis of avocado (*Persea americana* Mill.) and its application for plant improvement. *Acta Hort* 575:133–138
- Witjaksono, Litz RE (2004) Effect of gamma irradiation on embryogenic avocado cultures and somatic embryo development. *Plant Cell Tissue Organ Cult* 77:139–147
- Witjaksono, Litz RE, Grosser JW (1998) Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts. *Plant Cell Rep* 18:235–242
- Witjaksono, Litz RE, Pliego-Alfaro F (1999a) Somatic embryogenesis in avocado (*Persea americana* Mill.). In: Jain SM, Gupta PK, Newton RJ (eds) *Somatic embryogenesis in woody plants*, vol. 5. Kluwer, Dordrecht, pp 197–214
- Witjaksono, Schaffer B, Colls A, Litz RE, Moon PA (1999b) Avocado shoot culture, plantlet development and net CO<sub>2</sub> assimilation in an ambient and enhanced CO<sub>2</sub> environment. In *Vitro Cell Dev Biol-Plant* 35:238–244
- Zentmyer GA (1980) *Phytophthora cinnamomi* and the diseases it causes. American Phytopathological Society, St. Paul, Minneapolis
- Zentmyer GA, Paulus AO, Gustafson CD, Wallace JM, Burns RM (1965) Avocado diseases. *Calif Agric Exp Stn Ext Serv Circ* 534
- Zirari A, Lionakis SM (1994) Effect of cultivar, explant type, etiolation pretreatment and the age of plant material on the in vitro regeneration ability of avocado (*Persea americana*). *Acta Hort* 365:69–76

## I.8 Grape

A. PERL<sup>1</sup> and Y. ESHDAT<sup>1</sup>

### 1 Introduction

The use of breeding and genetics to boost crop productivity and quality, and the use of agricultural chemicals to protect crops and enhance plant growth, were the two prominent features of agriculture in the 20th century (Dandekar and Gutterson 2000). In the 21st century, in addition to provide necessary nutrients, crops are also expected to improve consumers' health. Integrating conventional technologies with those based on molecular biology and genomics could make this objective possible, since enhancing the desirable characteristics of agricultural crops, while reducing the expression of undesirable ones, is currently feasible for a variety of crops, including grape (Grant 2002).

#### 1.1 The Importance of Grapes

The Old World species *Vitis vinifera* is the grape of antiquity often mentioned in the Bible. Most table, wine and raisin grapes are produced from this species, the latter having originated in the regions bound between the south of the Caspian and Black Seas in Asia Minor (Perl and Eshdat 1998). Globally, grapevine (*Vitis* species) is the most economically important crop amongst fruit species. Due to the numerous uses of its fruit in producing wine, table grapes, dried fruit and organic compounds, it is also the most widespread fruit crop in the world. The world production of grapes is currently in excess of 85 million metric tons (Vivier and Pretorius 2000).

#### 1.2 The Need for Genetic Improvement of Grapes

The breeding constraints of grapevine were the prime motivation for the culture of grape cells as a means for developing transgenic approaches in this genus. Using conventional breeding, the time from the beginning of the selection procedure until the approval of a new variety takes approximately 10–15 years. Moreover, grapes are highly heterozygous and the characters that constitute a good cultivar are polygenic in their inheritance. Thus, the

<sup>1</sup> Department of Fruit Tree Sciences, Institute of Plant Science, Agricultural Research Organization, P.O. Box 6, 50250 Bet-Dagan, Israel, e-mail: perlx@int.gov.il

probability of recombining in a hybrid the desired set of genes, which determine the essential properties of a given cultivar, is very low.

It seems very likely that many, if not most, of the new properties that grape consumers may expect will be achievable through the application of genetic engineering. Though currently controversial in its application, it is strongly believed that genetic engineering will be invaluable to the future improvement of agricultural systems in general, and viticulture in particular.

## 2 Somatic Embryogenesis in Grapes

### 2.1 Embryonic Cell Cultures

In grapevine, the most widely used regeneration methods rely on embryogenic callus initiated from somatic cells of mainly anthers, anther filaments, ovules and, to a much lesser extent, cultured young leaves. The type and quality of this cell suspension is the key factor to successful transformation. Numerous review articles have described the protocols of the establishment, maintenance and use of somatic embryogenesis in grape (Perl and Eshdat 1998; Thomas et al. 2000; Vivier and Pretorius 2000; Colova-Tsolova et al. 2001; Kikkert et al. 2001; Martinelli and Garibaud 2001; Martinelli and Mandolino 2001; Bouquet et al. 2003; Perl et al. 2004). This technique was recently refined and extended to additional genotypes (Jayasankar et al. 1999, 2001; Martinelli et al. 2001a, b; Motoike et al. 2001), although major difficulties currently prevent its optimal utilization:

1. Grapevine's recalcitrance to tissue culture lies in poor embryogenic callus induction from differentiated tissues and in plant regeneration via embryogenesis, which is most often arrested at the late torpedo stage. Fine tuning of the media composition was shown to improve plant regeneration beyond the torpedo stage. Under such circumstances, genotypic differential responses were reduced (Perrin et al. 2001, 2004).
2. Many cultivars are still not amenable to genetic manipulations, which are strongly dependant on the genotype. However, the list of *Vitis* cultivars that have been introduced as embryogenic lines is constantly increasing (Gribaudo et al. 2000; Torregrosa et al. 2002b; Perl et al. 2004).
3. Zlenko et al. (2002) stressed the importance of the addition of gibberellic acid as a major factor to improve the germination and subsequent plant regeneration of grape somatic embryos. This assumption is based on the observation that chilling embryos at +4 °C for 4–8 months increased the concentration of biologically free gibberellin in these embryos.
4. Regeneration and germination of somatic embryos may be problematic as embryogenic cultures may age and accumulate mutations during subculture. Several studies have dealt with the long-term maintenance of the embryogenic potential of grape cultures. Size fractionation by ultrasound during



subcultures was found to be a major factor in maintaining actively growing morphogenetic cultures (Maitz 2000; Jayasankar and Bondada 2002). Using light and scanning electron microscopy, Jayasankar et al. (2003) compared the ontogeny of somatic embryos of grapevine produced from semi-solid and liquid culture-derived pro-embryogenic masses. Interestingly, this study illustrated that semi-solid-medium-derived somatic embryos exhibited dormancy, as do mature zygotic embryos, whereas liquid-medium-derived embryos did not show this phenomenon.

Recent studies (Gribaudo et al. 2003a, b; Popescu et al. 2003) have demonstrated the use of embryogenic cultures as a means to eliminate viruses from grape genetic stocks known to be infected by several grapevine viruses, including leaf roll-associated virus 3 (GLRaV-3).

Embryogenesis was also used as a tool to examine chimerism in grapevine. Frank et al. (2002) were able to show that separation by passage through somatic embryogenesis of the two cell layers, L1 and L2, of a Pinot Meunier periclinal chimera, yielded regenerated plants exhibiting novel phenotypes, which have distinct DNA profiles compared to those of the parent plant.

## 2.2 Cryopreservation of Grape Embryogenic Shoots and Cells

In vitro storage under growth-retarding conditions reduces the need for repeated subculture, which may lead either to the reduction of the morphogenetic capability or to premature germination of embryogenic lines. Slow growth was achieved by maintaining cultures in media supplemented with growth inhibitors, such as mannitol and flurprimol (Stevkov et al. 2003). Cryopreservation of embryogenic cells proved to be an efficient tool promoting embryogenesis and subsequent plant germination (Perl and Eshdat 1998; Wang et al. 2002). Wang and Perl (2005) have reviewed the recent advances in cryopreservation of grape embryogenic cell lines, the subsequent regeneration of plants from cryopreserved cells, and the superiority of cryopreserved cells for genetic transformation.

## 3 Selection Systems Utilizing Grape Cell Cultures

In vitro screening systems offered a promising approach for identification and isolation of disease-tolerant individuals, following the exposure of pro-embryogenic masses to filtrates of different pathogenic fungi and bacteria. However, regeneration of disease-resistant woody plants has rarely been achieved. Embryogenic cultures of 'Chardonnay' were exposed to culture filtrates of *Elsinoe ampelina* – the causal agent of anthracnose disease (Jayasankar et al. 2000; Jayasankar and Gray 2003). Putative resistant lines, specifically those able to inhibit the growth of *E. ampelina* and *Fusarium exysporium*, were further char-



acterized and found to secrete elevated concentrations of chitinase. Somatic embryos obtained from the selected lines grew without inhibition in a medium containing 40% fungal culture filtrate. Plants regenerated from these lines were shown to exhibit resistance to infection by *E. ampelina* in both glasshouse tests and detached leaf bioassays.

## 4 The International Grape Genome Program

A genomic approach to discover and determine the function of all grapevine genes is the most promising strategy to achieve a rapid and thorough understanding of grapevine biology. *Vitis* is well suited to genomic studies, having a genome size of approximately 500 Mbp. The general scientific goals of the current Grape Genome Project are to understand the genetic and molecular basis of all *Vitis* biological processes that are relevant to the crop. The knowledge acquired from the grape genome project will promote basic grape research in the fields of complex biological processes and evolutionary biology of grape. The specific objectives of the project can be viewed at <http://www.vitaceae.org>.

*Vitis* genomics research is currently addressing many viticulture and enology issues. The overall benefit of the application of genomics to grapevine will be an increase in the precision of genetic improvement and viticulture practices by establishing refined correlations between molecular characters and agricultural traits. The phenotype of a cultivated grape plant (berry quality, yield, vigor, biotic and abiotic stress tolerance) is the result of a dynamic and complex interaction between the environment, field management and genes of the cultivar (Ablett et al. 2000). Traits considered of fundamental interest are pathogen and biotic stress resistance, dormancy, the quality of table and wine grapes and reproduction.

Expressed sequence tags (EST) determined during the last 3 years have increased significantly the public-available sequence information for *V. vinifera*. The current National Center for Biotechnology Information (NCBI) *V. vinifera* dbEST database contains more than 147,000 ESTs. These ESTs were generated from 61 libraries using mainly the cultivars 'Chardonnay', 'Cabernet Sauvignon' and 'Syrah', representing a wide range of plant development stages, organs and genotypes, as well as grapevine challenges to various biotic and abiotic stresses. In order to identify disease-resistant- and stress-tolerant-related genes that may not exist in *V. vinifera* grapes, Lu and Hunter (2004) have sequenced over 13,000 ESTs from *Vitis shuttleworthii*, one of the most disease- and pest-resistant grape species. Specific data on grape ESTs can be viewed at <http://cgf.ucdavis.edu> and <http://www.tigr.org/tdb/tgi/vvgi> (Bohlman et al. 2004).

## 5 Genetic Transformation in Viticulture

In traditional viticulture, current genetic developments are concentrated on the production of disease- and pest-resistant varieties (Kikkert et al. 2001; Vivier and Pretorius 2002). Other approaches aim to accelerate ripening, enhance fruit quality and flavor, or induce parthenocarpy and seedlessness (Perl et al. 2000; Mezzetti et al. 2002). Some studies have focused on optimizing gene expression in transgenic grapes by screening for different constitutive promoters (Gollop et al. 2001, 2002; Li et al. 2001a; Torregrosa et al. 2002a), or selectable markers conferring resistance to kanamycin and paromomycin (Torregrosa et al. 2000a; Reustle et al. 2003a; Wang et al. 2004). Other studies used hygromycin and phosphinotrycin (Basta) as selective agents (Torregrosa et al. 2000b; Li et al. 2004), while mannose and xylose were found not to be useful as selectable agents for *Vitis* transformation (Semenzato et al. 2002; Keiffer et al. 2004). Recently, the superiority and elevated expression of novel bidirectional duplex promoters were demonstrated in grapevine somatic embryos. These promoters were constructed by placing two identical core promoters on both the upstream and downstream sides of their duplicated enhancer elements. Estimates of promoter function were obtained by monitoring GUS and enhanced-GFP in the transformed embryos (Li et al. 2004).

Up until 2004, 43 permits for field test releases had been granted for transgenic grapevines. Most tests were performed in the USA, with recent emphasis given to overcoming the bacterium-mediated Pierce's disease, which causes considerable damage in vineyards. Other field experiments were mainly performed in France, Italy, Germany (Harst et al. 2000), Canada and Australia (Locco et al. 2001). Table 1 summarizes the field experiments that involve transgenic grapevines (updated November 2004).

## 6 Utilization of Transgenic Grapes

Besides disease control, biotechnological approaches have focused more and more on fruit traits, such as sugar content (Davis and Boss 2000), color, seedlessness in table grapes and increased fruit yield (Vivier and Pretorius 2000). Stress- and cold-tolerant grape varieties have been developed using genes coding for either an antifreeze, superoxide dismutase (SOD) or the iron-binding protein ferritin (Tsvetkov et al. 2000; Gutoranov et al. 2001; Olah et al. 2004). Several noteworthy examples describing recent genetically engineered grape products aimed at acquiring pathogenic resistance or improved fruit quality are detailed below.

**Table 1.** Reported field experiments using transgenic grapevines – the state of the art (November 2004)

Country	Organization and cultivars	Inserted genes	Origin	Trait
Australia <sup>a</sup>	CSIRO	Polyphenol oxidase	Grapevine	Regulation of browning
	Shiraz	<i>sh4</i>	Grapevine	Regulates development of flowers and berries
	Sultana	<i>ufgt</i>	Grapevine	Regulation of anthocyanin synthesis
	Cabernet	<i>dfr</i>	Grapevine	Regulation of anthocyanin synthesis
	Sauvignon Chardonnay	<i>inv</i> <i>gfp</i>	Grapevine <i>Aequorea victoria</i>	Increases sucrose levels Monitors pollen flow
Italy <sup>b</sup>	University of Ancona	Tryptophan-2-monooxygenase		Synthesis of auxin
France <sup>c</sup>	INRA, Colmar rootstocks	Genes against grapevine fan-leaf nepovirus	Viral sources	Virus resistance
Germany <sup>d</sup>	Geilweilerhof Dornfelder Riesling Seyal Blanc	Chitinase Glucanase RIP	Not specified	Fungal resistance
USA <sup>e</sup>	University of California	$\beta$ -glucuronidase Polygalacturonase inhibitor protein	<i>E. coli</i> Pear	Marker gene Fungal resistance
	Cornell	Magainin	<i>Xenopus laevis</i>	<i>Xyllela</i> resistance
	University	Antimicrobial peptide	<i>Amaranthus caudatus</i>	Powdery mildew resistance
		Antimicrobial peptide Chitinase	Synthetic <i>Trichiderma harzianum</i>	<i>Botrytis</i> resistance Powdery mildew resistance
USA	New York state	Lignan biosynthesis	Pea	Powdery mildew resistance
	Geneseo	$\beta$ -1,3 -glucanase	Pea	Powdery mildew resistance
	Anton Caratan	RNAase	Unknown	Seedlessness
	AgriVitis	Confidential Business Information	Unknown	<i>Agrobacterium</i> resistance <i>Nepovirus</i> resistance Crown gall resistance <i>Closterovirus</i> resistance

Table 1. (continued)

Country	Organization and cultivars	Inserted genes	Origin	Trait
	GenApps	Confidential Business Information	Unknown	<i>Nepovirus</i> resistance <i>Closterovirus</i> resistance
Canada	University of Guelph	SOD	<i>Arabidopsis thaliana</i>	Cold tolerance

<sup>a</sup> Application for licensing for international release of a genetically modified organism (GMO) into the environment: application no. DIR 031/2002, Office of the Gene Technology Regulator, Australia

<sup>b</sup> Deliberate field trials, environmental releases of GMO;  
<http://biotech.jrc.it/delibetare/it.asp>

<sup>c</sup> Deliberate field trials, environmental releases of GMO;  
<http://biotech.jrc.it/delibetare/fr.asp>

<sup>d</sup> Deliberate field trials, environmental releases of GMO;  
<http://biotech.jrc.it/delibetare/de.asp>

<sup>e</sup> Information System for Biotechnology, field test release permits database;  
<http://www.isb.vt.edu/cfdocs/fieldtests3.cfm>

## 6.1 Viral Resistance

Grapevine virus infection can cause severe losses by substantially reducing yield, affecting fruit quality and shortening the lifespan of plants in the vineyard. Genetic manipulation is an attractive way to insert specific virus-resistant genes into commercial elite cultivars (Fuchs 2003). The most commonly genes used for transformation studies include those encoding the viral coat protein (CP), movement protein or RNA-dependent RNA polymerase. Significant tolerance was achieved against grapevine virus A (GVA) and grapevine virus B (GVB) and *Arabis* mosaic nepovirus (ArMV) (Martinelli et al. 2000; Radian-Sade et al. 2000; Spielmann et al. 2000). Some of the introduced sequences were in the form of antisense or untranslatable versions (Golles et al. 2000; Mauro et al. 2000; Gutoranov et al. 2001). *V. vinifera* cv. *Arich dresse* was transformed with a construct containing the inverted repeats of the grapevine fanleaf virus (GFLV) movement protein (Jardak-Jamoussi et al. 2003). Gribaudo et al. (2003a) introduced nine different constructs containing chimeric CP genes of GFLV, either as full-length, truncated forms or as untranslatable forms of the CP gene, in either antisense or sense orientation.

Krastanova et al. (2000) reported in detail the obtaining of virus resistance by transforming embryogenic cultures of several rootstocks. The gene construct included translatable, antisense or non-translatable CP genes from GFLV, GLRaV-3 and GLRaV-2. A gene sequence in an open reading frame of GLRV-3 was also used. Various levels of tolerance to GFLV were observed

by different inoculation techniques including micrografting, heterografting with GFLV-infected *Chenopodium quinoa*, in vitro green grafting and woody grafting.

A recent novel approach to obtaining virus resistance in transgenic grapes is based on utilizing antibodies capable of binding to the viral replicases, movement proteins or CP. Transgenic grapes expressing specific antibodies against the CP of GLRaV-3 are currently under evaluation for their viral tolerance (Fischer and Schillberg 2003; Nolke et al. 2003).

Another promising new approach to the introduction of virus resistance in grapes is based on post-transcriptional gene silencing, a natural process during which steady state levels of mRNA are diminished by targeted degradation. By expressing dsRNA complementary to gene fragments of GLRaV-3 and grapevine rupestris stem pitting-associated virus (GRSPaV) in grapevine, Burger et al. (2003) aimed to acquire resistance to these two viruses. Reustle et al. (2003b) obtained viral gene silencing using several constructs and showed one to three copies of the introduced genes in the transgenic grapes. One of the major environmental safety issues concerning transgenic crops containing virus-derived genes relates to the outcome of recombination events between viral transgene transcript and RNAs from the indigenous virus population. Vigne et al. (2004) addressed this issue. Over a 3-year period, transgenic grapevines neither promoted the development of viable GFLV recombinants to detectable levels nor affected the genetic diversity of indigenous GFLV populations.

## 6.2 Bacterial Resistance

The most significant pathogen in viticulture in the USA is the bacterium *Xylella fastidiosa*, the causative agent of Pierce's disease (PD). Merlot and Chardonnay grapes, transformed with the synthetic version of the ceropin antimicrobial gene (Li et al. 2001b; Scorza and Gray 2001), were inoculated with *Xylella* and exhibited different level of symptoms of PD. However, these transformants exhibited peptide concentrations in the xylem sap below that required for *Xylella* tolerance (Li et al. 2001a, b).

Vidal et al. (2003) utilized the bombardment technique to co-transform Chardonnay plants with different antimicrobial genes under the control of either the double 35S cauliflower mosaic virus or the ubiquitin promoter. Co-transformation was carried out using either the natural or the synthetic magainin-2 lytic peptide, fused in frame to different signal peptides, to direct secretion into the cell cytosol.

In another attempt to obtain resistance to PD, Meredith (2001) produced transgenic Chardonnay carrying a gene that codes for a pear polygalacturonase inhibitor protein (PGIP) and two derivatives of ribosome-inactivating protein (RIP) from *Trichosanthes kirilowii*, labeled with GFP-protein either in its amino- or carboxyl-terminal. Western blot analysis demonstrated the presence of the PGIP protein in roots, leaves and young stems of transgenic plants.

Selected transgenic lines showed delayed development of PD (Meredith 2001; Aguero et al. 2003).

Crown gall is another major bacterial disease affecting grapes worldwide and is transferred by wild-type *Agrobacterium vitis*. Holden et al. (2003) transformed embryos with a truncated *virE2* gene encoding VirE2 protein lacking 215 amino acids from its carboxyl terminus. This region is predicted to be associated with DNA binding, thus preventing these grapevines from becoming re-infected in the field.

### 6.3 Fungal Resistance

The pathogenic fungi downy mildew, botrytis and powdery mildew are the main targets for production of fungal-tolerant transgenic grapes. Reisch et al. (2003) were among the first to use an endochitinase-producing gene (ThEn42) from *Trichoderma*, as well as two magainin-type antimicrobial peptide genes (*MagII* and *MSI99*), to achieve fungal disease tolerance in transgenic Chardonnay and Merlot plants. Increased chitinase (10- to 100-fold) was observed by Western blot analysis in 40–50% of both in vitro and field-grown transformed plants. In a detached leaf assay, the lesion size after *Botrytis* infection was 65% less in the transgenic line expressing a high chitinase concentration compared to the control. Six out of 25 transgenic Chardonnay lines exhibited increased tolerance towards powdery mildew after natural inoculation in the field (Kikkert et al. 2003). Transgenic grape containing pathogen-related genes from barley or rice that encode chitinases and/or glucanases, both known as cell wall-degrading enzymes, showed enhanced resistance against powdery mildew caused by *Uncinula necator*, as well as some resistance against *E. ampelina* (Jeandet et al. 2002).

Besides pathogen-resistant (PR) proteins, accumulation of phytoalexins such as stilbenes is the other major defense mechanism frequently observed and well characterized in grapevine (Jeandet et al. 2002; Ferreira et al. 2004). Stilbene production is controlled by a key enzyme, stilbene synthase, which produces trans-resveratrol, the major phytoalexin in grape (Montero et al. 2003). A chimeric gene, combining a fungal-inducible promoter (alfalfa PR-10) with a grape stilbene synthase gene (*Vst 1*), was introduced into the rootstock 41B. These transgenic plants exhibited reduced symptoms following in vitro inoculation with *Botrytis cinerea*, probably due to the accumulation of resveratrol at concentrations up to 100-fold above the control (Coutos-Thevenot et al. 2001).

Eutypine is a toxin produced by the fungus *Eutypa lata*. Inactivation of its toxicity is enzymatically possible via its conversion to eutypinol by an NADPH-dependent aldehyde reductase. The gene encoding this enzyme in *Vigna radiate*, designated as *Vr-ERE*, was isolated recently. Transgenic 110R plants overexpressing *Vr-ERE* increased their detoxification capacity and enhanced their resistance to *E. lata* (Amborabe et al. 2000; Legrand et al. 2003).

Ribosome-inhibiting proteins are able to block fungal ribosomes and are also considered capable of blocking fungal growth. The *Run1* gene, which was isolated from *Muscadinia rotundifolia*, was able to confer total resistance to powdery mildew, and transgenic *Vinifera* plants expressing this gene are under evaluation for fungal disease tolerance (Bouquet et al. 2000).

## 6.4 Fruit Quality

Most consumers prefer seedless varieties of grapes, a demand that dominates the market place. Therefore, studies were launched to explore novel strategies to convert seeded grapes to seedless using biotechnological approaches. Koltunow et al. (1998) were the first to prevent the formation of the hard seed-coat layer, yet allowing normal development of fruit and seeds. A later approach has been based on directed overexpression of auxins in the fertilized ovule. Such overproduction in ovules just before fertilization was shown to stimulate the production of parthenocarpic seedless fruits (Rotino et al. 1997; Perl et al. 2000; Mezzetti et al. 2002).

# 7 Recent Advances in Grape Molecular Biology

## 7.1 Flower Induction and Development

The transition from vegetative to reproductive growth in grapevine is an essential and critical stage in the life cycle of the plant. Grape flowers are unusual in their structure and development. The inflorescences are initiated during spring time in latent buds, formed in the axil of each leaf. Limited inflorescence development occurs during the same growing season until dormancy. In the next spring, inflorescences undergo further maturation and development. The number of inflorescences per vine and the number of flowers per inflorescence have a major impact on fruitfulness, yield and berry quality. Boss et al. (2003) reviewed recent developments in flower induction, meristem and organ identity in grapes. An understanding of the function of the key genes controlling floral induction and development may pave the way for improving vineyard fruit production.

Joly et al. (2004) isolated grape homologues to those of *Arabidopsis* flowering genes, namely *LEAFY*, *APETALA1*, *AGAMOUS*, *TERMINAL FLOWER1* and *SEPALLATA3*. Expression of *VvLEAFY* and *VvTFL1* was detected early after germination in the shoot apical meristem of seedlings and later in the latent buds of juvenile plants. However, it was not sufficient to induce flowering, which occurs only after 3–4 years of vegetative development. A similar pattern of expression was observed for the meristematic-expressed *VFL* gene (Carmona et al. 2002). In adult plants, continuous gene expression of *VvLEAFY* was evident over two growing seasons. In the following spring, only latent



buds that developed into flower-bearing shoots showed expression of *VvTFL1*, *VvLEAFY*, *VvAPI*, *VvAG* and *VvSEP3*, suggesting that their expression was required for flower ontogenesis. Boss and Thomas (2002) elegantly demonstrated that gibberellins inhibit flowering in grapevine. A grapevine dwarf mutant was utilized, which was able to produce inflorescences along the length of the shoot where tendrils normally develop. The mutated gene associated with this phenotype was shown to be a homologue of the wheat 'green revolution' gene and the *Arabidopsis* gene GA insensitive (*GAI*). The conversion of tendrils to inflorescences demonstrated that the grapevine tendril is a modified inflorescence inhibited from completing floral development by gibberellins. Other molecular support for the hypothesis that *Vitis* tendrils are modified reproductive organs adapted for climbing comes from the study by Calonje et al. (2004), who reported the isolation of two MADS-box genes, *VFUL-L* and *VAPI*. The MADS-box family genes play a key role in flower and fruit development and are involved in the differentiation of the floral meristem into the various floral organs. Four other MADS-box cDNAs had been previously cloned by Boss et al. (2001, 2002) from grapevine flowers and unripe berries. Two of these grape clones were related to the *SEPALLATA* genes, a third showed homology to *AGAMOUS-LIKE 6* and 13, and the fourth to *AGL11*. All clones were expressed in fully developed flowers. The expression of the *AGL11* homologue was restricted to the carpel and was found to be expressed only in female flowers. The expression pattern of these genes suggests that they have a role in the regulation of both grapevine flower and berry development. The recently isolated *VFUL-L* and *VAPI* were both expressed in lateral meristems that, in grapevine, can give rise to either inflorescence or tendrils. During flower development, *VFUL-L* transcripts are restricted to the central part of young flower meristems and, later, to the prospective carpel-forming region. The expression pattern of *VAPI* suggests involvement in flower transition and development. Both are expressed throughout tendril development, independent of the flowering process.

## 7.2 Berry Ripening

Recent genomic studies, demonstrating changes taking place during berry ripening (Davis and Robinson 2000), yielded ripening-associated cDNA libraries derived from a Shiraz berry (Venter et al. 2000). In berries at the onset of ripening, a rapid increase occurred in the mRNA levels of a number of cDNAs not present in unripe fruit. The putative translation products of some of these clones seemed to be involved in cell wall structure. These included proline-rich proteins and two other glutamate-rich proteins. The remainder of the clones encoded putative stress response proteins including thaumatin, metallothionein, cytochrome P450 and proteins induced by water, sugar and/or cold stress. Burger et al. (2004) supplied additional evidence that grape proline-rich proteins are associated with ripening. 'Merlot' ripening-induced protein 1 (*meip1*) was shown to be specifically transcribed in the fruit tissue, seed

and bunchstems from veraison until final ripening. Accumulation was limited specifically to the mesocarp and exocarp cells of the ripening berry.

### *7.2.1 Sucrose Metabolism During Berry Ripening*

Grape berries, when ripe, contain high concentrations of sugars that are important for flavor and fermentation. The accumulation of sugars in grape berries begins at the onset of ripening, and is marked by an increase in mainly glucose and fructose accumulating mostly in the cell vacuole. Manning et al. (2001) expressed two putative ripening-related sucrose transporters from grape berries in an invertase-deficient yeast strain. The results of this study suggested that these grape transporters may facilitate the loading of sucrose from the apoplast into the cells.

Or et al. (2000b) isolated and characterized a cDNA clone encoding for a grape pyruvate decarboxylase (PDC) and characterized its expression throughout berry development. The pattern of PDC gene expression suggested that PDC is not the limiting factor for the production of ethanol in the berry. Alternatively, the induction of alcohol dehydrogenase gene expression, which occurs only after the onset of ripening, may serve as a regulator of ethanol production in response to a ripening-related cue.

The ASR (ABA, stress and ripening-induced) proteins were induced during ripening of grape berries and seem to be involved in plant development. Cakir et al. (2003) isolated a grape ARS gene named *VvMSA*. Its expression was upregulated at different stages of berry ripening and was induced by sucrose and strongly enhanced by ABA. *VvMSA* was shown to bind specifically to the promoter of the putative monosaccharide transporter (*VvHT1*) and, more precisely, to two sucrose-responsive elements present on this fragment. Demonstration of a positive regulation of *VvHT1* promoter activity by *VvMSA* indicates that *VvMSA* acts as part of a transcription-regulation complex involved in sugar signaling.

Another interesting gene with unknown function in the grape berry was found to encode a thaumatin-like protein (*VVTL1*). Southern, Northern and Western analyses revealed that *VVTL1* is found only in the berry, and its single gene is expressed in conjunction with the onset of sugar accumulation and softening. The timing of its accumulation correlates with the inability of the fungal pathogen powdery mildew to initiate new infections of the berry and may hint on its function.

### *7.2.2 Antifungal Genes During Berry Development*

Fungi often cause serious damage to grape berries during their development, leading to consequent losses in vintage quality and quantity. Chitinases constitute a group of defense molecules for which direct activity against pathogens has been demonstrated. The involvement of chitinases during grape berry

development and the interaction between ripening berries and fungi were studied by Robert et al. (2002). Three genomic sequences encoding chitinases were isolated. Two of them were shown to belong to class I chitinases with a putative vacuolar (*Vvchit1a*) and extracellular (*Vvchit1b*) localization, while the third belongs to class III (*VvchitIII*). Transcripts of the class III chitinase were shown to accumulate in unripe berries after infection with *Plasmopara viticola*, but not in the later developmental stages of berries infected either with this fungus or with *B. cinerea*. Class I chitinases were never detected in berries.

### 7.2.3 Pigmentation of Developing Berries

Anthocyanins, which are the predominant pigments in red and black grape berries, are important compounds for red wine making as well as table grapes. Northern blot analysis revealed that mRNAs, other than *UDP-glucose:flavonoid 3- $\beta$ -glucosyltransferase* (*Ufgt*), are active in the early developing stage of the berry and decreased until veraison. Later, mRNA of all genes increased during the coloring stages of only red cultivars (Jeong et al. 2004). Three genomic clones of chalcon synthase (*Chs1–Chs3*) were obtained and their accumulation in grape berry skin was compared. RT-PCR analysis showed that the mRNA of *Chs3* accumulated mainly in the berry skin of red cultivars during coloration, while the mRNA of *Chs1* and *Chs2* accumulated in the leaves and berry skin of white as well as red cultivars, thus indicating that the three *Chs* genes seemed to be under different transcription control (Goto-Yamamoto et al. 2002). On the other hand, the mRNA of *Ufgt* was detected only in red cultivars (Kobayashi et al. 2001) and the introduction of *Ufgt* cDNA into colorless embryos induced reddish-purple spots (Kobayashi et al. 2002). Differences in the regulation of flavonol synthase (FLS) were reported by Downey et al. (2003) by comparing the expression of two cDNA clones (*VvFLS1* and *VvFLS2*) isolated from flowers. *VvFLS1* was expressed in leaves, tendrils, pedicels, buds and inflorescences as well as in developing grapes. Expression was highest between flowering and fruit set, then it declined, and it increased again during ripening. Expression of the *VvFLS2* was much less than *VvFLS1* and did not change during berry development.

Anthocyanin accumulation was enhanced by ABA and suppressed by auxins, high temperature and low light intensity, as was mRNA accumulation of the *VvmyA1*, a putative regulatory gene of anthocyanin biosynthesis. Treatment of grape berries with the ethylene-releasing compounds at veraison was shown to increase the levels of *Chs* and *F3H* transcripts. Transcript levels of *LDOX* and *Ufgt* were similarly enhanced, but to a lesser extent. The transcript accumulation of dihydroflavonol 4-reductase (*DFR*) was unaffected (El-Kereamy et al. 2003). Low doses of ethanol were also shown to enhance anthocyanin accumulation in addition to stimulating the maturation of grape berries. While the expression of most genes in the anthocyanin pathway was inhibited or unchanged by ethanol treatment, there was a marked increase in the expression

of *Ufgt*, suggesting that this gene is a key factor in anthocyanin accumulation following ethanol treatment (El-Kereamy et al. 2002).

### 7.3 Grape Dormancy

Grape dormancy is initiated by decreasing photoperiod or temperature. This is considered as a cue to signal the end of the growing season and to initiate physiological, developmental and molecular changes necessary to enter a state of dormancy required to survive winter extremes. The spraying of hydrogen cyanamide (HC) is a horticultural dormancy-breaking procedure. Or et al. (2002) identified several genes that were induced as a result of HC and were shown to be involved in dormancy release. Grape catalase was shown to decline sharply following induction of dormancy release by HC. A protein kinase (GDBRPK), an SNF-like protein kinase, was found to be involved in the perception of the stress signal induced by HC application and may be created following possible induction of oxidative stress (Or et al. 2000a). In parallel, induction of transcripts for the enzymes pyruvate decarboxylase and alcohol dehydrogenase following HC application was exemplified. The transient induction detected in both transcript levels, usually induced only following the development of a respiratory disturbance, may imply that HC application indeed leads to a transient respiratory stress.

## 8 Conclusions

Grapes are among other gene-revolutionary plant products to be recently developed using 'genetic engineering' techniques. Such techniques have invoked considerable controversies over food safety, as well as consumer and political resistance to genetically modified organisms.

We believe that genetic engineering will prove to be invaluable to the future improvement of *Vitis*. The given phenotype of a grape cultivar (berry, quality, yield, vigor and more) is a result of complex interactions between the environment, management regime and the genotype of the cultivar. Integration of the scientific approaches described in this chapter may contribute to the successful incorporation of transgenes of interest into grape, paving the way for improving rootstock and variety performance.

## References

- Ablett E, Seaton G, Scott K, Shelton D, Graham MW, Baverstock P, Lee LS, Henry R (2000) Analysis of grape ESTs: global gene expression patterns in leaf and berry. *Plant Sci* 159:87–95
- Agüero C, Dandekar A, Meredith C (2003) Evaluation of tolerance to Pierce's disease and botrytis in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. *Acta Hort* 603:473–478

- Amborabe BE, Fleurat-Lessard P, Bonmort J, Roustan JP, Robin G (2000) Effects of eutypine, a toxin from *Eutypa lata*, on the plant cell plasma membrane. *Plant Physiol Biochem* 38:51–58
- Bohlman M, Ergul A, Tattersall EAR, Tillett RL, Figueroa R, Kabuloglu E, Cushman MA, Spreeman KL, Schlauch K, Mendes P, Cramer G, Cushman JC (2004) Microarray gene expressing profiling studies of abiotically stressed grapevine (*Vitis vinifera* L.). *Proc Plant Biology 2004 Symp*, American Society of Plant Biologists, Florida, Abstract no. 899
- Boss PK, Thomas MR (2002) Association of dwarfism and floral induction with a grape 'green revolution' mutant. *Nature* 416:847–850
- Boss PK, Vivier M, Matsumoto S, Dry IB, Thomas MR (2001) A cDNA from grapevine (*Vitis vinifera* L.), which shows homology to *AGAMOUS* and *SHATTERPROOF*, is not only expressed in flowers but also throughout berry development. *Plant Mol Biol* 45:541–553
- Boss PK, Sensi E, Hua C, Davies C, Thomas MR (2002) Cloning and characterization of grapevine (*Vitis vinifera* L.) MADS-box genes expressed during inflorescence and berry development. *Plant Sci* 162:887–895
- Boss PK, Buckeridge EJ, Poole A, Thomas MR (2003) New insights into grapevine flowering. *Funct Plant Biol* 30:593–606
- Bouquet A, Pauquet J, Adam-Blondon AF, Torregrosa L, Merdinoglu D, Wiedemann-Merdinoglu J (2000) Towards the obtention of grapevine varieties resistant to downy mildews by conventional breeding and biotechnology. *Bull OIV* 73:445–452
- Bouquet A, Torregrosa L, Chatelet P (2003) Grapevine genetic engineering: tool for genome analysis or plant breeding method? Which future for transgenic vines? *AgBiotechNet* 5(116):1–10
- Burger AL, Zwiegelaar JP, Botha FC (2004) Characterization of a gene encoding the Merlot ripening-induced protein 1 (*mrip1*): evidence that this putative protein is a distinct member of the plant praline-rich protein family. *Plant Sci* 167:1075–1089
- Burger JT, Engelberg M, Van Eeden C (2003) The construction of gene silencing vectors for the introduction of multiple virus resistance in grapevine. *Proc 14th ICVG Conf*, Locorotondo, pp 225–227
- Cakir B, Agasse A, Gaillard C, Saumonneau A, Delrot S, Atanassova R (2003) A grape ASR protein involved in sugar and abscisic acid signaling. *Plant Cell* 15:2165–2180
- Calonje M, Cubas P, Martinez-Zapater M, Carmona MJ (2004) Floral meristem identity genes are expressed during tendrill development in grapevine. *Plant Physiol* 135:1491–1501
- Carmona MJ, Cubas O, Nartinez-Zapater M (2002) VFL, the grapevine *FLORICULA/LEAFY* ortholog, is expressed in meristematic regions independently of their fate. *Plant Physiol* 130:68–77
- Colova-Tsolova V, Perl A, Krastanova S, Tsvetkov I, Atanassov A (2001) Genetically engineered grape for disease and stress tolerance. In: Roubelakis-Angelakis KA (ed) *Molecular biology and biotechnology of the grapevine*. Kluwer, Dordrecht, pp 411–432
- Coutos-Thenot P, Poinssot B, Bonomelli A, Year H, Breda C, Buffard D, Esnault R, Hain R, Boulay M (2001) In vitro tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase *Vst1* gene under the control of a pathogen-inducible PR 10 promoter. *J Exp Bot* 52:901–910
- Dandekar AM, Gutterson N (2000) Genetic engineering to improve quality, productivity and value of crops. *Calif Agric* 54:49–56
- Davis C, Boss PK (2000) The use of molecular biology techniques to study and manipulate the grapevine: why and how? *Aust J Grape Wine Res* 6:159–167
- Davis C, Robinson SP (2000) Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. *Plant Physiol* 122:803–812
- Downey MO, Harvey JS, Robinson SP (2003) Synthesis of flavonols and expression of flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Aust J Grape Wine Res* 9:110–121

- El-Kereamy A, Chervin C, Souquet JM, Moutounet M, Monje MC, Npveu F, Mondies H, Ford MC, van Heeswijck R, Roustan JP (2002) Ethanol triggers grape berry expression leading to anthocyanin accumulation during berry ripening. *Plant Sci* 163:449–454
- El-Kereamy A, Chervin C, Roustan JP, Cheynier V, Souquet JM, Moutounet M, Raynal J, Ford MC, Latche A, Pech JC, Bouzayen M (2003) Exogenous ethylene stimulates the long term expression of genes related to anthocyanin biosynthesis in grape berries. *Physiol Plant* 119:175–182
- Ferreira RB, Monteiro SS, Antonieta PP, Teixeira AR (2004) Engineering grapevine for increased resistance to fungal pathogens without compromising wine quality. *Trends Biotechnol* 22:168–173
- Fischer R, Schillberg S (2003) Engineering durable resistance in grapevines: a novel strategy for integrated disease management to overcome environmental impact of pesticides. *Proc 14th ICVG Conf, Locorotondo*, p 224
- Frank T, Botta R, Thomas MR (2002) Chimerism in grapevine: implication for cultivar identity, ancestry and genetic improvement. *Theor Appl Genet* 104:192–199
- Fuchs M (2003) Transgenic resistance: state of the art and perspectives. *Proc 14th ICVG Conf, Locorotondo*, pp 221–223
- Golles R, Moser R, Puhlinger H, daCamara Machado ML, Minafra A, Savino V, Saldarelli P, Martelli GP, daCamara Machado A (2000) Transgenic grapevines expressing coat protein gene sequences of grapevine fanleaf virus, arabis mosaic virus A and grapevine mosaic virus B. *Acta Hort* 528:305–311
- Gollop R, Farhi S, Perl A (2001) Regulation of the leucoanthocyanidin dioxygenase gene expression in *Vitis vinifera*. *Plant Sci* 161:579–588
- Gollop R, Even S, Colova-Tsolova V, Perl A (2002) Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. *J Exp Bot* 53:1397–1409
- Goto-Yamamoto N, Wan GH, Masaki K, Kobayashi S (2002) Structure and transcription of three chalcone synthase genes of grapevine (*Vitis vinifera*). *Plant Sci* 162:867–872
- Grant EI (2002) Agricultural biotechnology. In: Grant EI (ed) *Agricultural biotechnology and transatlantic trade: regulatory barriers to GM crops*. CAB International, Wallingford, Oxon, pp 31–55
- Gribaudo I, Vallania R, Franks T, Miaja ML, Thomas M (2000) Genotype influence on somatic embryogenesis in grapevine anther and leaf culture. *Acta Hort* 528:407–410
- Gribaudo I, Scariot V, Gambino G, Schubert A, Golles R, Laimer M (2003a) Transformation of *Vitis vinifera* L. cv Nebbiolo with coat protein gene of Grapevine Fanleaf Virus (GFLV). *Acta Hort* 603:309–314
- Gribaudo I, Bondaz J, Cuoizzo D, Gambino G (2003b) Elimination of grapevine leafroll-associated virus 3 from the wine grapevine Muller-Thurgau (*Vitis vinifera* L.) through somatic embryogenesis. *Proc 14th ICVG Conf, Locorotondo*, pp 240–241
- Gutoranov G, Tsvetkov I, Colova-Tsolova VM, Atanassov AI (2001) Genetically engineered grapevines carrying GFLV coat protein and antifreeze genes. *Agric Consp Sci* 66:69–74
- Harst M, Bornhoff BA, Zyprian E, Topfer R (2000) Influence of culture technique and the genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. *Vitis* 39:99–102
- Holden M, Krasatanova S, Xue B, Pang S, Sekiya M, Momol D, Gonsalves D (2003) Genetic engineering of grape for resistance to crown gall. *Acta Hort* 603:481–484
- Jardak-Jamoussi R, Bouamama B, Krczal G, Mliki T, Wetzel T, Reustle GM, Ghorbel A (2003) Regeneration of Tunisian transgenic grapevine plants and evaluation of the gene construct for grapevine fanleaf virus resistance. *Proc 14th ICVG Conf, Locorotondo*, pp 248–249
- Jayasankar S, Bondada BR (2002) A unique morphotype of grapevine somatic embryogenesis exhibits accelerated germination and early plant development. *Plant Cell Rep* 20:907–911
- Jayasankar S, Gray DJ (2003) In vitro selection for disease resistance in plants – an alternative to genetic engineering. *AgBiotechNet* 5(111)
- Jayasankar S, Gray DJ, Litz RE (1999) High-efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. *Plant Cell Rep* 18:533–537



- Jayasankar S, Li Z, Gray DJ (2000) In vitro selection of *Vitis vinifera* 'Chardonnay' with *Elsinoe ampelina* culture filtrate is accompanied by fungal resistance and enhanced secretion of chitinase. *Planta* 211:200–208
- Jayasankar S, Van Aman M, Li Z, Gray DJ (2001) Direct seeding of grapevine somatic embryos and regeneration of plants. *In Vitro Cell Dev Biol-Plant* 37:476–479
- Jayasankar S, Bhaskar R, Bondada R, Li Z, Gray DJ (2003) Comparative anatomy and morphology of *Vitis vinifera* (Vitaceae) somatic embryos from solid- and liquid-culture derived proembryogenic masses. *Am J Bot* 90:973–979
- Jeandet P, Douillet-Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M (2002) Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *J Agric Food Chem* 50:2731–2741
- Jeong ST, Goto-Yamamoto N, Kobayashi S, Esaka M (2004) Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Sci* 167:247–252
- Joly D, Perrin M, Gertz C, Kronenberger J, Demangeat G, Masson JE (2004) Expression analysis of flowering genes from seedling-stage to vineyard life of grapevine cv. Riesling. *Plant Sci* 166:1427–1436
- Keiffer F, Triouleyre C, Bertsch C, Farine S, Leva Y, Walter B (2004) Mannose and xylose cannot be used as selectable agents for *Vitis vinifera* L. transformation. *Vitis* 43:35–39
- Kikkert J, Ali GS, Wallace PG, Reisch B, Reustle GM (2003) Expression of a fungal chitinase in *Vitis vinifera* L. 'Merlot' and 'Chardonnay' plants produced by biolistic transformation. *Acta Hort* 528:297–303
- Kikkert JR, Thomas MR, Reisch BI (2001) Grapevine genetic engineering. In: Roubelakis-Angelakis KA (ed) *Molecular biology and biotechnology of grapevine*. Kluwer, Dordrecht, pp 393–463
- Kobayashi S, Ishimaru M, Ding CK, Yakushiji H, Goto N (2001) Comparison of UDP-glucose:flavonoid 3-O-glucosyltransferase (UGT) gene sequences between white grape (*Vitis vinifera*) and their sports with red skin. *Plant Sci* 160:543–550
- Kobayashi S, Ishimaru M, Hiroka K, Honda C (2002) *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. *Planta* 215:924–933
- Koltunow AM, Brennan P, Bond JE, Barker SJ (1998) Evaluation of genes to reduce seed size in *Arabidopsis* and tobacco and their application to *Citrus*. *Mol Breed* 4:235–251
- Krastanova S, Ling KS, Zhu HY, Xue B, Burr TJ, Gonsalves D (2000) Development of transgenic grapevine rootstocks with genes from grapevine fanleaf virus and grapevine leafroll associated closterovirus 2 and 3. *Acta Hort* 528:367–372
- Legrand V, Dalmayarc S, Latche A, Pech JC, Bouzayen M, Fallot J, Torregrosa L, Bouquet A, Roustan JP (2003) Constitutive expression of *Vr-ERE* gene in transformed grapevines confers enhanced resistance to eutypine, a toxin from *Eutypa lata*. *Plant Sci* 164:809–814
- Li Z, Jayasankar S, Gray DJ (2001a) Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). *Plant Sci* 160:877–887
- Li Z, Jayasankar S, Gray DJ (2001b) An improved enzyme-linked immunoabsorbent assay protocol for the detection of small lytic peptides in transgenic grapevines (*Vitis vinifera*). *Plant Mol Biol Rep* 19:341–351
- Li Z, Jayasankar S, Gray DJ (2004) Bi-directional duplex promoters with duplicated enhancers significantly increase transgene expression in grape and tobacco. *Transgenic Res* 13:143–154
- Locco P, Franks T, Thomas MR (2001) Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Res* 10:105–112
- Lu J, Hunter W (2004) Towards identifying Pierce's disease resistant genes from a native American grape species (*Vitis shuttleworthii*) – a genomic approach. In: Tariq MA, Oswalt S, Blincoe P, Lorick A, Esser T (eds) *Pierce's Disease Research Symp Proc*, San Diego, California, pp 31–34
- Maitz M (2000) Use of an ultrasound cell retention system for the size fractionation of somatic embryos of woody species. *Plant Cell Rep* 19:1057–1063



- Manning K, Davies C, Broen HC, White PJ (2001) Functional characterization of two ripening related sucrose transporters from grape berries. *Ann Bot* 87:125–129
- Martinelli L, Garibaud I (2001) Somatic embryogenesis in grapevine (*Vitis* spp.). In: Roubelakis-Angelakis KA (ed) *Molecular biology and biotechnology of grapevine*. Kluwer, Dordrecht, pp 327–352
- Martinelli L, Mandolino G (2001) Transgenic transformation in *Vitis*. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 47. *Transgenic crops II*. Springer, Berlin Heidelberg New York, pp 325–338
- Martinelli L, Costa D, Poletti V, Festi S, Buzkan N, Minafra A, Saldarelli P, Martelli GP, Perl A (2000) Genetic transformation of tobacco and grapevine for resistance to viruses related to rugose wood disease complex. *Acta Hort* 528:321–327
- Martinelli L, Candioli E, Costa D, Poletti V, Rascio N (2001a) Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. *Plant Cell Rep* 20:279–284
- Martinelli L, Garibaud I, Bertoldi D, Candioli E, Poletti V (2001b) High efficiency embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo. *Vitis* 40:111–115
- Mauro MC, Coutos-Thevenot P, Boulay M, Valat L, Barbier P, Walter B, Pinck L (2000) Analysis of 41B (*Vitis vinifera* × *Vitis berlandieri*) grapevine rootstocks for grapevine fanleaf virus resistance. *Acta Hort* 528:313–319
- Meredith C (2001) Genetic transformation to improve the Pierce's disease resistance of existing grape varieties. In: 2000–2001 Annual Report, American Vineyard Foundation, California Competitive Grant Program in Viticulture and Enology, pp 1–4
- Mezzetti B, Pandolfini T, Navacchi O, Landi L (2002) Genetic transformation of *Vitis vinifera* via organogenesis. *BioMed Central Biotechnol* 2:18
- Montero C, Cristescu SM, Jimenez JB, Orea JM, Hekkert L, Harren FJM, Urena AG (2003) trans-Resveratrol and grape disease resistance. A dynamical study by high-resolution laser-based techniques. *Plant Physiol* 131:129–138
- Motoike SY, Skirvin RM, Norton MA, Otterbacher AG (2001) Somatic embryogenesis and long term maintenance of embryogenic lines from fox grapes. *Plant Cell Tissue Organ Cult* 6:121–131
- Nolke G, Orecchia M, Saldarelli R, Dell'Orco M, Minafra A, Martelli GP, Fischer R, Schillberg S (2003) Antibody-based resistance in grapevine: generation, characterization and expression of single chain antibody fragment specific to grapevine leafroll associated virus 3. *Proc 14th ICVG Conf, Locorotondo*
- Olah R, Toth A, Ruthner S, Korbuly J, Szegedi E (2004) Genetic transformation of rootstock cultivar Richter 110 with the gene encoding the ironbinding protein, ferritin. *Acta Hort* 652:471–473
- Or E, Vilozny I, Fennel A, Eyal Y, Ogrudovitch A (2000a) The transduction of the signal for grapebud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. *Plant Mol Biol* 43:483–489
- Or E, Baybik J, Sadka A, Ogrudovitch A (2000b) Fermentative metabolism in grape berries: isolation and characterization of pyruvate decarboxylase cDNA and analysis of its expression throughout berry development. *Plant Sci* 156:151–158
- Or E, Vilozny I, Fennel A, Eyal Y, Ogrudovitch A (2002) Dormancy in grape buds: isolation and characterization of catalase cDNA and analysis of its expression following chemical induction of bud dormancy release. *Plant Sci* 162:121–130
- Perl A, Eshdat Y (1998) DNA transfer and gene expression in transgenic grapes. In: Tombs MP (ed) *Biotechnology and genetic engineering reviews*, vol. 15. Intercept, Andover, pp 365–386
- Perl A, Sahar N, Spiegel-Roy P, Gavish S, Elyassi R, Or E, Bazak H (2000) Conventional and biotechnological approaches in breeding seedless table grapes. *Acta Hort* 528:607–612
- Perl A, Colova-Tsolova V, Eshdat Y (2004) Agrobacterium-mediated transformation of grape embryogenic calli. In: Curtis IS (ed) *Transgenic crops of the world – essential protocols*. Kluwer, Dordrecht, pp 229–242

- Perrin M, Martin D, Joly D, Demangeat G, This P, Masson JE (2001) Medium-dependent response of grapevine somatic embryogenic cells. *Plant Sci* 161:107–116
- Perrin M, Gertz C, Masson JE (2004) High efficiency initiation of regenerable embryogenic callus from anther filaments of 19-grapevine genotypes grown worldwide. *Plant Sci* 167:1343–1349
- Popescu CF, Buciumeanu EC, Visofu E (2003) Somatic embryogenesis, a reliable method for grapevine fleck virus free grapevine regeneration. *Proc 14th ICVG Conf, Locorotondo*, p 243
- Radian-Sade S, Perl A, Edelbaum O, Kuzentsova L, Gafny R, Sela I, Tanne E (2000) Transgenic *Nicotiana benthamiana* and grapevine plants transformed with grapevine virus A (GVA) sequences. *Phytoparasitica* 28:79–86
- Reisch BI, Kikkert J, Vidal J, Ali GS, Gadoury D (2003) Genetic transformation of *Vitis vinifera* to improve disease resistance. *Acta Hort* 603:303–308
- Reustle GM, Wallbraun M, Zwiebel M, Wolf R, Manthey T, Burkhardt C, Lerm T, Vivier M, Krczal G (2003a) Selectable marker system for genetic engineering of grapevine. *Acta Hort* 603:485–490
- Reustle GM, Jarda-Jamoussi R, Ebel R, Burkhardt C, Becker M, Wolf R, Manthey T, Bassler A, Wetzel T, Ghorbel A, Krczal G (2003b) Induction of silencing in transgenic tobacco (*Benthamiana*) and grapevine (*Vitis* spp.) plants. *Proc 14th ICVG Conf, Locorotondo*, p 228
- Robert N, Roche K, Lebeau Y, Colette B, Boulay M, Esnault R, Buffard D (2002) Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. *Plant Sci* 162:389–400
- Rotino GL, Perri E, Zottini M, Sommer H, Spena A (1997) Genetic engineering of parthenocarpic plants. *Nat Biotechnol* 15:1398–1401
- Scorza R, Gray DJ (2001) Disease resistance in *Vitis*. US patent no 6,232528. Mid-Florida Research and Education Center, University of Florida
- Semenzato M, Poletti V, Martinelli L (2002) The use of phosphomannose isomerase as a selectable marker to transfer foreign genes in grape (*Vitis* spp.). *Proc 46th SIGA Annu Congr, Italian Society of Agricultural Genetics, Giardini Naxos*, Abstract 1.21
- Spielmann A, Krastanova S, Douet-Orhand V, Gugerli P (2000) Analysis of transgenic grapevine (*Vitis vinifera*) and *Nicotiana benthamiana* plants expressing an Arabis mosaic virus coat protein gene. *Plant Sci* 156:235–244
- Stevkov I, Ionnou N, Hadjinicoli A, Atanassov A (2003) Flurprimidol and mannitol as tool for in vitro storage of grapevine germplasm. *Proc 14th ICVG Conf, Locorotondo*, pp 248–249
- Thomas MR, Locco P, Franks T (2000) Transgenic grapevines: status and future. *Acta Hort* 528:279–284
- Torregrosa L, Lopez G, Bouquet A (2000a) Antibiotic sensitivity of grapevine: a comparison between the effect of hygromycin on shoot development of transgenic 110 Richter rootstock (*Vitis Berlandieri* × *Vitis rupestris*). *S Afr J Enol Vitic* 21:32–39
- Torregrosa L, Peros JP, Lopez G, Bouquet A (2000b) Effects of hygromycin, kanamycin and phosphinothricin on the embryogenic callus development and axillary micropropagation of *Vitis vinifera* L. *Acta Hort* 528:401–406
- Torregrosa L, Verrios C, Tesniore C (2002a) Grapevine (*Vitis vinifera* L.) promoter analysis by biolistic-mediated transient transformation of cell suspension. *Vitis* 41:27–32
- Torregrosa L, Locco P, Thomas MR (2002b) Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. *Am J Enol Vitic* 53:183–190
- Tsvetkov IJ, Atanassov AI, Tsoleva VM (2000) Gene transfer for stress resistance in grapes. *Acta Hort* 528:389–394
- Venter M, Burger AL, Botha FC (2000) Molecular analysis of fruit ripening: the identification of differentially expressed sequences in *Vitis vinifera* using cNDA-AFLP technology. *Vitis* 40:191–196
- Vidal JR, Kikkert JR, Wallace PG, Reisch BI (2003) High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing *npt-II* and antimicrobial peptide genes. *Plant Cell Rep* 22:252–260

- Vigne E, Komar V, Fuchs M (2004) Field safety assessment of recombination in transgenic grapevines expressing the coat protein gene of Grapevine fanleaf virus. *Transgenic Res* 13:165–179
- Vivier MA, Pretorius IS (2000) Genetic improvement of grapevine: tailoring grape varieties for the third millennium. *S Afr J Enol Vitic* 21:5–26
- Vivier MA, Pretorius IS (2002) Genetically tailored grapevines for the wine industry. *Trends Biotechnol* 20:472–478
- Wang Q, Perl A (2006) Cryopreservation of embryogenic cell suspensions by encapsulation-vitrification. *Meth Mol Biol* 318:77–86
- Wang Q, Gafny R, Sahar N, Mawassi M, Tanne E, Perl A (2002) Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions and subsequent plant regeneration by encapsulation-dehydration. *Plant Sci* 162:551–558
- Wang Q, Mawassi M, Sahar N, Li P, Colova-Tsolova V, Gafny R, Sela I, Tanne E, Perl A (2004) Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification. *Plant Cell Tissue Organ Cult* 77:267–275
- Zlenko VA, Kotikov IK, Troshin LP (2002) Efficient GA<sub>3</sub> plant regeneration from cell suspensions of three grape genotypes via somatic embryogenesis. *Plant Cell Tissue Organ Cult* 70:295–299

## I.9 Melon

J.C. PECH<sup>1</sup>, A. BERNADAC<sup>1</sup>, M. BOUZAYEN<sup>1</sup>, A. LATCHE<sup>1</sup>,  
C. DOGIMONT<sup>2</sup>, and M. PITRAT<sup>2</sup>

### 1 Introduction

Edible melons (*Cucumis melo* L.) are an important horticultural crop in tropical and subtropical regions, but they are also grown extensively in temperate countries. According to the FAO ([www.fao.org](http://www.fao.org)), world production of cantaloupes and other melons in 2005 was about 28 million tons, with around 70% cultivated in Asia (Table 1). Because of the wide diversity of the family Cucurbitaceae, melons represent only about 15% of the total production of cucurbits (164 million tons) and of this, approximately 70% (129 million tons) is cultivated in Asia. When compared with the production of other juicy tropical fruit, melons rank fourth (with over 27 million tons) after citrus (over 100 million tons), watermelons (over 90 million tons) and bananas (around 70 million tons), before mangoes, pineapples and papayas (Table 2). Thirty two species of the genus *Cucumis* have been recognised (Kirkbride 1993). The genus can be divided into the subgenus *Cucumis* with only two species, namely the domestic cucumber (*Cucumis sativus* L.) and wild cucumber (*C. hystrix* Chakravarty), both of Asian origin, and the subgenus *Melo* with 30 species, including the cultivated melon (*C. melo* L.) of African origin. Within the subgenus *Melo*, *C. anguria* L. and *C. metuliferus* Meyer (ex Naudin) are also cultivated, but on a smaller scale than melon or cucumber. *C. sativus* has a haploid chromosome number ( $n$ ) of 7, while all the other species have  $n = 12$ , some of which are tetraploids or hexaploids.

Wild melons with small, round or oval fruits, 20–50 g in weight, are found commonly in Africa. Probably, originally, like many other cucurbitaceous members such as watermelon (*Citrullus*) and squash (*Cucurbita*), melon was domesticated for its seeds because the fruit flesh was bitter. In Sudan, the *seinat* type is still cultivated for seeds. According to their use, melon cultivars can be divided into three main groups: those in which the fruits are not sweet at maturity, but are harvested at the immature stage and eaten raw, pickled or cooked, fruits that are harvested at maturity and are sweet, and fruits that are not edible but are used for their fragrance. Some rare cultivars of melon are cultivated for their leaves in southern Africa (Vorster and Jansen van Rensburg 2004).

<sup>1</sup> INRA/INPT-ENSAT “Génomique et Biotechnologie des Fruits” (UMR 990), Av. de l’Agrobiopole, BP 32607, 31326 Castanet-Tolosan Cedex, France, e-mail: [pech@ensat.fr](mailto:pech@ensat.fr)

<sup>2</sup> INRA, Unité de Génétique et Amélioration des Fruits et Légumes, BP 94, 84143 Avignon Cedex, France

**Table 1.** Production of cantaloupes and other melons compared with total cucurbits in different regions of the world in 2005 (<http://www.faostat.fao.org/>)

		Africa	Asia	Europe	North + Central America	South America	World
Cantaloupes	Million tons	1.45	20.50	3.18	2.48	0.65	28.26
+ other melons	Million ha	0.067	0.91	0.14	0.13	0.05	1.30
Cucurbits	Million tons	7.96	129.04	14.45	9.74	3.50	164.69
	Million ha	0.65	6.46	0.78	0.58	0.25	8.72

**Table 2.** World production of cantaloupes and other melons compared with that of other major fresh tropical fruit in 2004 (<http://www.fao.org/>)

Rank	Type of fruit	Production (million tons)	Area (million ha)
1	Citrus	108.09	7.39
2	Watermelon	93.48	3.46
3	Banana	70.63	4.55
4	Cantaloupes + other melons	27.40	1.31
5	Mango	26.29	3.70
6	Pineapple	15.29	0.84
7	Papaya	6.50	037

Jeffrey (1980) proposed dividing *C. melo* into two subspecies according to the hairiness of the ovary, namely subsp. *agrestis* with short hairs, found throughout India and eastern Asia, and subsp. *melo* with long hairs, found throughout India, central and western Asia, Europe and the New World (Table 3). The two subspecies could correspond to independent domestications. Wild melons may have short or long pubescence of the ovary.

Within these two subspecies, different botanical groups or *varietas* have been defined according to fruit traits. Naudin's classification (Naudin 1859) into ten *varietas* has formed the basis for later works. A better knowledge of melon from central Asia and the Far East has resulted in more *varietas*. For instance, *momordica* is characterised by a mealy non-sweet flesh at maturity, a very thin fruit epidermis and the bursting of the fruit at maturity. It is found only in India. *Flexuosus* has long to very long fruits (up to 2 m), known by the names of snake melon, snake cucumber, fakouss, adjour and kakri, which are eaten raw in salads. It is cultivated throughout Morocco and Egypt, Turkey, Iraq and India. *Conomon* resembles, and is eaten like, a cucumber and is cultivated in China, Korea and Japan. Those in the *cantalupensis* grouping are defined by their round ribbed fruits with orange flesh and typical aroma. The fruits of the *reticulatus* group have characteristic netting on the fruit epidermis. These latter two groups are merged by some authors into the *cantalupensis* group.

**Table 3.** *Varietas* of melon according to subspecies (Jeffrey 1980) and use of the fruit

Characteristics	Subsp. <i>agrestis</i>	Subsp. <i>melo</i>
'Vegetable' (non-sweet), usually harvested immature	<i>conomon</i>	<i>chate</i>
	<i>momordica</i>	<i>flexuosus</i>
	<i>acidulus</i>	<i>tibish</i> and <i>seinat</i>
'Fruit' (sweet), usually harvested at maturity	<i>makuwa</i>	<i>cantalupensis</i>
	<i>chinensis</i>	<i>reticulatus</i>
		<i>inodorus</i>
		<i>adana</i>
		<i>chandalak</i>
'Fragrance', non edible, harvested at maturity		<i>ameri</i>
		<i>dudaim</i>

Melons of the *inodorus* group are characterized by their non-climacteric fruits with low aroma. The fruits can be very sweet, and some have a long shelf life. The cultigroups Cassaba, Tendral, Amarillo, Honeydew, Piel de sapo and Kirkagac belong to the *inodorus*. *Dudaim* is an original botanical group with fruits the size of an orange; they are not sweet, but have a very strong typical aroma. They are cultivated in Asia, throughout Turkey and Afghanistan.

Numerous attempts have been made, using conventional pollination methods, to generate interspecific crosses between *C. melo* and other *Cucumis* species, mainly *C. metuliferus* and *C. sativus*. In spite of some published results (Norton and Granberry 1980; Fanourakis 1988; Lebeda et al. 1997), no horticultural trait has been transferred from one melon species to another species. In this respect, biotechnological methods offer the possibility to overcome these difficulties and to introduce novel characters that cannot be introgressed by traditional breeding. This chapter reports on the new developments in melon biotechnology in terms of in vitro regeneration by organogenesis or somatic embryogenesis, of haploid production and genetic transformation. Applications to the improvement of disease resistance, fruit quality and storability and other agronomic characters are also discussed. Other reviews on melon and cucurbit biotechnology have been published in recent years (see Guis et al. 1998; Bernadac et al. 2002).

## 2 Molecular Tools, Markers and Mapping

*C. melo* has a small genome size, estimated at 450–500 Mbp for the haploid genome (1C) (Arumanagathan and Earle 1991), similar to that of rice and 3.5 times that of the model plant *Arabidopsis thaliana*. Melon has a chloroplast genome estimated at 150 kb (Palmer 1982; Perl-Treves and Galun 1985)

and a large mitochondrial genome of 2,400 kb (Ward et al. 1981). Maternal transmission of the chloroplast and paternal transmission of the mitochondrial genome have been demonstrated (Havey et al. 1998). Cultivated melon is diploid with  $2n = 2x = 24$  chromosomes. Haploid, triploid or tetraploid plants have been obtained (see Sect. 5). Melon chromosomes are small in size and cytogenetic studies are not easy to conduct. In spite of some attempts (Ramachandran and Seshadri 1986; Dane 1991; Ma et al. 1994), it is still proving difficult to obtain a good karyotype of melon. Until now, stable monosomic or trisomic plants have not been identified.

## 2.1 Biochemical and Molecular Markers

A “marker” can be defined as a polymorphic trait with a simple inheritance, i.e., a Mendelian segregation. The polymorphism can be phenotypic (e.g. flower biology, disease resistance), biochemical (isozymes) or detectable at the DNA level using different methods such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellites (SSR), intermicrosatellites (ISSR) or single nucleotide polymorphism (SNP).

Biochemical and molecular polymorphisms have been investigated since the 1980s, first with isozymes (Esquinas Alcazar 1981; Dane 1983; Perl-Treves et al. 1985; Sujutha et al. 1991; Meglic et al. 1994; Akashi et al. 2002; Kato et al. 2002; McCreight et al. 2004) and then with molecular markers (Neuhausen 1992; Katzir et al. 1996; Garcia et al. 1998; Stepansky et al. 1999; Garcia-Mas et al. 2000; Staub et al. 2000; Mliki et al. 2001; Akashi et al. 2002; Baba et al. 2002; Decker-Walters et al. 2002; Liu et al. 2002; López-Sesé et al. 2002, 2003; Monforte et al. 2003; Staub et al. 2004; Nakata et al. 2005; Szabo et al. 2005; Yashiro et al. 2005). Comparisons made between isozymes and RAPD (Staub et al. 1997), between RAPD and SSR (Staub et al. 2000; López-Sesé et al. 2002), or between RAPD, AFLP and RAPD (Garcia-Mas et al. 2000) were generally in good agreement, although with some discrepancies between the different types of markers. Intraspecific classification and relationship with the *varietas* has been investigated using biochemical and molecular markers. In general, the different accessions belonging to the different *varietas* fell in the same cluster determined by the molecular markers, but there were many discrepancies. The largest divergence seemed to be between the non-sweet *momordica*, *flexuosus*, *conomon*, *dudaim* and wild melons, and, on the other hand, the sweet *inodorus*, *cantalupensis* and *reticulatus* (Stepansky et al. 1999). In many cases there were fewer differences between cultigroups than between accessions of different geographic origin belonging to the same cultigroup (Mliki et al. 2001; López-Sesé et al. 2002). Until now, molecular markers have not been used for the management of melon genetic resources, for instance to define core collections, as has been suggested for cucumber with isozymes and morphological traits (Staub et al. 2002).



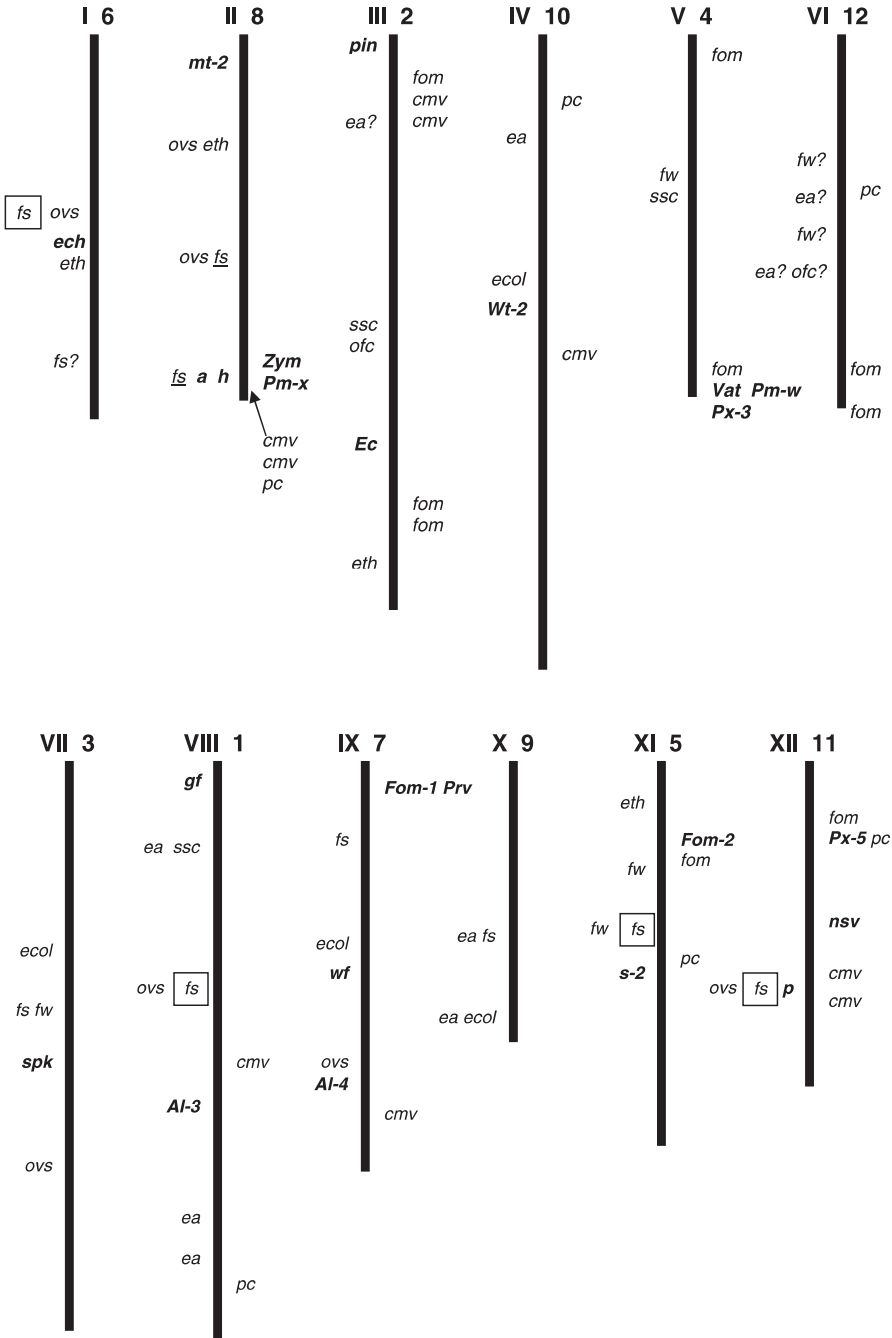
Divergent parents for molecular and phenotypical characters are the basis to establishing genetic maps (see Sect. 2.2). It has also been suggested that the genetic diversity could be extended by using accessions with extensive molecular polymorphism (Mliki et al. 2001; López-Sésé et al. 2002). However, a clear relationship has not been established between molecular polymorphism and the horticultural value of cultivars or heterosis.

Biochemical or molecular markers are used by seed companies to fingerprint the cultivar. They are also used in quality control to check purity and detect inbreds in  $F_1$  seed production.

## 2.2 Genetic Maps and Synteny

One of the most promising uses of molecular markers is in the development of genetic maps and subsequent marker assisted selection. Melon is a highly polymorphic species according to fruit traits and disease resistance. Inheritance of some of these characters has been studied and many genes and quantitative trait loci (QTL) have been described. Some of these have been located on genetic maps. An initial genetic map was published by Pitrat (1991) with only phenotypic traits involving many crosses. Subsequently, Baudracco-Arnas and Pitrat (1996), Wang et al. (1997), Oliver et al. (2001), Danin-Poleg et al. (2002), Périn et al. (2002b), Silberstein et al. (2003) and Gonzalo et al. (2005) presented maps using different types of molecular markers and localized some traits on these maps. Several parents have been used to build these maps, even if some lines are common to several maps, for instance, the multi-resistant accessions PI 161375 or PI 414723. The size of the map depends on, amongst other factors, the type of progeny. For example, it has been estimated to 1,200 cM on an  $F_2$  (Oliver et al. 2001), 1,950 cM on a back-cross (Wang et al. 1997) and 1,650 cM on two recombinant inbred line progenies (Périn et al. 2002b). The construction of a consensus map by merging these complementary maps has yet to be conducted, probably by using SSR as anchor points (Fig. 1). Recently, a collection of 57 near-isogenic lines have been obtained, each one containing a unique independent introgression from PI 161375 in a Piel de Sapo genetic background (Eduardo et al. 2005). This population is an interesting tool, especially for the mapping of complex traits.

Synteny between related species allows the use of information from one species to be used for tagging orthologous genes in another species. Amongst the cucurbits, the melon map is the most advanced. Cucumber has a small genome and  $x = 7$  chromosomes, but has a low level of polymorphism, and divergence between parents is not enough to easily build a good map. Watermelon and *Cucurbita* genetic maps are still of poor quality. In a first attempt to use SSR as common markers between melon and cucumber, nine markers were polymorphic. Linkage group B in cucumber seems to correspond to linkage groups E and 2 of melon (Danin-Poleg et al. 2000). The comparative mapping of the Zucchini yellow mosaic virus (ZYMV) resistance in melon and cucumber



◀ **Fig. 1.** A tentative synthetic map of melon genes (*bold characters*) and QTL (*normal characters*). Names of the linkage group (LK) are in *roman numerals* (Périn et al. 2002b) and *arabic numbers* (Oliver et al. 2001). Due to some doubts concerning alignment of the two maps (top and bottom), QTL with a *questionmark* on LG I, III and VI could be in other positions. On the *right side* of each linkage group are genes or QTL for disease resistance and on the *left side* are genes or QTL for other traits (flower biology, fruit characters). Genes for disease resistance: *Fom* *Fusarium oxysporum melonis*; *nsv* Melon necrotic spot virus; *Pm* powdery mildew; *Prv* Papaya ringspot virus; *Px* *Podosphaera xanthii* (powdery mildew); *Vat* Virus aphid transmission resistance; *Zym* Zucchini yellow mosaic virus. QTL for disease resistance: *cmv* Cucumber mosaic virus; *fom* *Fusarium oxysporum melonis* race 1.2; *pc* *Pseudoperonospora cubensis* (downy mildew). Genes for other traits: *a* andromonoecious; *Al* Abscission layer; *Ec* Empty cavity; *ech* exaggerated curvature of the hook; *gf* green flesh; *h* halo cotyledons; *mt* mottled (spots) fruit; *p* pentamerous; *pin* pine seed shape; *s* suture; *spk* speckled fruit epidermis; *wf* white flesh; *Wt* white seed. QTL for other traits: *ea* earliness; *ecol* external colour of the fruit; *eth* ethylene; *fs* fruit shape; *fw* fruit weight; *ofc* orange fruit colour; *ovs* ovary shape; *ssc* soluble solid contents. QTL for fruit shape described by Périn et al. (2002a) are *underlined*, those described by Monforte et al. (2004) are in *roman* characters, and those that could be in common are in *boxes*. (Data from Dogimont et al. 2000; Brotman et al. 2002; Périn et al. 2002a,b,c; Monforte et al. 2004; Perchepped et al. 2005a,b)

showed that these two regions were non-syntenic (Park et al. 2004b). Microsynteny was found between a sequenced melon bacterial artificial chromosome (BAC) clone on linkage group 4 (= LGV) and *A. thaliana* chromosomes 3 and 5 (van Leeuwen et al. 2003).

### 2.3 Marker Assisted Selection

The genes or QTLs mapped so far represent only a small proportion of the total genes involved in the huge phenotypic variation of melon. Nevertheless, markers linked to genes or QTL of horticultural values can be used in marker assisted selection (MAS). This general strategy was first defined for other crops and is now used in melon by seed companies to trace monogenic traits, such as disease resistance. The number of molecular markers available for MAS is increasing rapidly (Park et al. 2004a; Brotman et al. 2005; Morales et al. 2005; Noguera et al. 2005). So far, QTLs for disease resistance or fruit quality have not been characterized. One of the main problems with the practical use of genetic markers in breeding programs is the recombination events between a marker and the gene of interest. This is particularly true when a gene has been used for a long time and introduced into elite germplasm in several cultigroups. Markers found in one particular genetic background may not be linked to the locus of interest in another background. Therefore, markers need to be defined for every elite genotype or designed within the coding gene, when it has been isolated.

## 2.4 Molecular Tools and Positional Cloning

BAC libraries have been developed and used for positional cloning or synteny studies (Luo et al. 2001; van Leeuwen et al. 2003; Park et al. 2004b). An EST library with 3731 sequences (2467 unigenes) can be consulted online (<http://melon.bti.cornell.edu/>). Another EST library has been used to identify single nucleotide polymorphism (Morales et al. 2004). The first step for positional cloning is the fine mapping of a region with a gene of interest. This strategy is illustrated in Sect. 3 for the cloning of *Vat*, *Fom-2* and *nsv* genes.

## 3 Molecular Characterization of Resistance Genes

More than 40 loci have been described in melon that confer resistance to viruses, fungi and insects (Pitrat 2002). Most of these are dominant (*Zym*, *Prv*, *Fom-1*, *Fom-2*, *Pm*, *Vat*) and some of them display recessive inheritance, mostly those conferring resistance to viruses (*nsv*, *cab-1* and *cab-2*). Few have been cloned, indicating that the melon genome is suitable for a map-based cloning strategy. The small size of the melon genome (450 Mb) and the availability of a saturated genetic map with an average physical/genetic distance ratio of 280 kb/cM are favourable features for map-based cloning of targeted genes. Taking advantage of high-throughput methods of DNA extraction and genotyping, fine genetic mapping is presently in progress (A. Bendahmane et al., personal communication). For generating high-resolution physical maps, large DNA insert libraries have been constructed in BACs from different multiresistant melon genotypes, including MR-1 (Luo et al. 2001), a double haploid line PIT92 obtained from a cross with PI 161375 (van Leeuwen et al. 2003), PI 161375, WMR29 and PI 124112 (Pauquet et al. 2004; A. Bendahmane, personal communication). These new resources will greatly accelerate the cloning of new resistance genes and their characterization in terms of molecular variability within the species. They will open up new prospects for exploiting the natural diversity of melon through the screening of a large number of accessions for additional alleles at a particular locus. This is expected to lead to the isolation of genes that may confer greater or broader resistance.

Two dominant resistance genes have been cloned: specifically, (1) the *Vat* gene, which confers resistance to an aphid species, *Aphis gossypii*, and to the transmission by this vector of unrelated viruses such as Cucumber mosaic virus (CMV) and ZYMV (Pauquet et al. 2004); and (2) the *Fom-2* gene, which confers resistance to the soil-borne fungus *Fusarium oxysporum* f.sp. *melonis* races 0 and 1 (Joobeur et al. 2004). They were shown to belong to a large family of resistance genes encoding proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains, which control resistance to a wide variety of pathogens and pests in various plant species (Dangl and Jones 2001; Martin et al. 2003). Both melon genes belong to the second subclass of

NBS-LRR genes, which code for proteins containing putative coiled-coil (CC) domain.

The *Vat* gene has been isolated by a map-based cloning strategy. Two segregating populations have been used. A population of 200 recombinant inbred lines (RIL) obtained from the cross between the charentais line Védraçais and the resistant Korean line PI 161375 has been characterized for aphid resistance and used to saturate the region with molecular markers. A 6000 back cross (Védraçais  $\times$  PI 161375)  $\times$  Védraçais population was screened for recombination events within an interval of 1.7 cM delimited by markers flanking the *Vat* locus, which allowed the construction of a fine genetic map of the region. Recombinant plants were evaluated for resistance to aphid colonization and virus transmission and no recombination event has been observed between these two phenotypes. Markers tightly linked to and flanking *Vat* were used to screen a BAC library obtained from the homozygous resistant PI 161375. Using markers generated from the end sequences of the selected BACs, a physical interval containing a single gene was delimited by recombination events. Thus, a single gene was demonstrated to activate plant responses, which include mechanisms that modify the behaviour and the traits of life of *A. gossypii* and also suppress the multiplication of unrelated viruses. The isolated gene, 5.9 kb in length, carries five exons and four introns and encodes a predicted 1473-amino acid protein, expected to be localized in the cytoplasm. A specific feature of the *Vat* gene is the presence of four repeated motifs of 65 amino acids, extremely conserved within the LLR domain. A paralog of *Vat* has been identified and it has a very high identity to *Vat* but does not confer any known resistance phenotype. This *Vat* paralog lacks one of the four repeated motifs of 65 amino acids, suggesting a role of this domain in the specificity of the recognition mechanisms requested for resistance. Complementation experiments conducted by transferring an 11-kb genomic fragment carrying the *Vat* gene under the transcriptional control of its own promoter into the susceptible Védraçais melon yielded transformants resistant to aphid colonization and to virus transmission by *A. gossypii* (Pauquet et al. 2004).

The *Fom-2* gene has also been cloned by a map-based cloning strategy using the same RIL (Védraçais  $\times$  PI 161375) population. Two additional populations have been derived from two F7 RILs segregating for *Fom-2*. A BAC contig was built from the MR-1 library (Luo et al. 2001) and the sequencing of two overlapping partial BAC clones identified three candidate genes. Efforts were concentrated on one of these because of its high similarity to resistance genes of the NBS-LRR class. The putative *Fom-2* is 3 kb long and contains an uninterrupted open reading frame predicted to encode a 1073-amino acid polypeptide. Nucleotide sequences analysis revealed a large variation between the resistant MR-1 genotype and susceptible varieties (Védraçais, Ananas Yokneam and Durango) within the LRR domain of this putative *Fom-2* gene. Moreover, susceptible breeding lines were shown to have a large rearrangement in the LRR region. Nevertheless, complementation experiments have not been conducted to validate the function of this gene.

Both the *Vat* and *Fom-2* genes belong to clusters of homologue resistance genes (Brotman et al. 2002). A gene located at 17 kb from *Vat* in the same orientation, with 80% amino acid identity and truncated genes highly homologous to some parts of the *Vat* gene have also been identified in the cluster (Pauquet et al. 2004). In contrast, a close copy of *Fom-2* was not found in the vicinity of the locus, but a resistance gene homologue (RGH) was located 0.7 cM from *Fom-2* (Brotman et al. 2002; Joobeur et al. 2004). Both genomic regions contained several sequences with similarity to transposable elements, which are thought to have a major implication in the evolution of resistance genes (Joobeur et al. 2004; Pauquet et al. 2004).

The recessive *nsv* gene, which confers complete resistance to Melon necrotic spot virus (MNSV; a fungus-borne virus of the *Carmovirus* genus), corresponds to the eIF4E gene belonging to the complex of RNA translation initiation factors. The isolation of this gene was obtained by a combination of a map-based cloning strategy and a candidate gene strategy (Morales et al. 2005; Nieto et al. 2006). This gene family is involved in natural resistance to viruses, mainly potyviruses, in different crop species such as pepper, lettuce and pea (Ruffel et al. 2002; Nicaise et al. 2003; Gao et al. 2004). The mutation of a single nucleotide in the 3' end of the gene, resulting in the change of an amino acid, differentiates resistant and susceptible melons to MNSV.

Two genes, *At1* and *At2*, associated with resistance to the oomycete pathogen *Pseudoperonospora cubensis*, responsible for downy mildew in melon, have been cloned by reverse genetics (Taler et al. 2004). They encode the photorespiratory peroxisomal enzymes, glyoxylate aminotransferases, involved in basal defence pathways, and showed constitutive expression in resistant and susceptible melon plants, but at a much lower level in the susceptible ones. Transgenic melon plants over-expressing either of these genes displayed enhanced activity of glyoxylate aminotransferases and resistance to *P. cubensis*. The very high degree of identity between the coding sequences of the susceptible and resistant genotypes suggests that resistance is mediated by regulatory factors controlling the level of expression of these genes (Taler et al. 2004).

## 4 In Vitro Regeneration by Organogenesis and Somatic Embryogenesis from Callus and Protoplasts

Efficient in vitro regeneration protocols are essential for successful and reproducible genetic transformation. A number of protocols for the regeneration of melon via organogenesis, embryogenesis and from protoplasts have been established during the last two decades. However, melon is still considered as being difficult to regenerate and there is a large dependence on the genotype for the capacity to regenerate. Some of the basic methodological principles are discussed here, with emphasis on recent findings.

## 4.1 Organogenesis

Shoot formation can be induced directly from cotyledon, leaf or hypocotyl explants. In cotyledons, there is a polarity for shoot formation. It occurs preferentially at the basal region probably in relation to the preferential accumulation of growth factors, such as auxins (Leshem 1989). The age of cotyledons is critical. Young cotyledons are more frequently used for both better regeneration efficiency and reducing endo-polyploidization. The ploidy level of regenerated plants is considered as being correlated with the ploidy level of the explant source. It is therefore preferable to use quiescent cotyledons from mature seeds or shortly after germination, if polyploids are to be avoided (Guis et al. 1998). Endo-polyploidization results in altered phenotype, including reduced productivity of the plants and fruit that is not marketable due to its flat shape and small size.

Bud formation can also be initiated from leaf explants. When the explant is taken from plants grown *in vitro*, very young leaves are generally preferred (Kathal et al. 1988). In a *C. melo* var *cantalupensis* type (cv. Védraçais), the use of leaf explants led to a greater regeneration frequency and more regenerated diploid plants than the use of cotyledons (Guis et al. 2000).

Direct regeneration from hypocotyls had not been reported until Curuk et al. (2002) described a method capable of regenerating multiple shoots on MS medium (Murashige and Skoog 1962) in the presence of 4.4  $\mu\text{M}$  benzyladenine (BA). Regeneration depended on the presence of a fragment of the cotyledon remaining attached to the hypocotyl and it occurred through the formation of new shoot apical meristems in the proximal part of the hypocotyl. Regeneration from the hypocotyl is described as much more rapid than the more commonly reported regeneration from cotyledonary explants, producing shoots within 2 weeks compared to more than a month required for cotyledon explants. In addition, it resulted in nearly 100% diploid shoots in the dark or in the light (Curuk et al. 2003). Regeneration can also be achieved from callus of cotyledons (Moreno et al. 1985), hypocotyls (Kathal et al. 1986) and roots (Kathal et al. 1994). However, the age of the original tissue is critical, as demonstrated for root callus (Kathal et al. 1994).

The hormone balance for shoot organogenesis consists of an auxin/cytokinin ratio of less than 1, although sometimes only cytokinins are used (Dong et al. 1991; Curuk et al. 2002). BA is the cytokinin most frequently employed for inducing shoot formation. The presence of another growth regulator, such as ancymidol, an antigibberellin, has been reported to hasten bud development in cotyledonary explants of *Galia* melons (Gaba et al. 1996). Enhancement of multiple shoot induction in cotyledons of melon has been achieved in MS medium containing BA in combination with adenine sulphate and L-glutamine (Muruganatham et al. 2002). Shoot elongation requires reduction of cytokinin with eventual addition of gibberellins (Curuk et al. 2002), while rooting can be achieved in a hormone-free medium or in the presence of low concentrations of auxin (Guis et al. 2000). The presence of ethylene in the culture vessel reduces



shoot formation from cotyledons. Addition to the medium of an inhibitor of ethylene action, silver thiosulfate (STS), can counteract this effect (Roustan et al. 1992), but in further steps, STS is deleterious to root formation (unpublished data). Cotyledons from melon plants, in which ethylene production has been suppressed by an antisense ACC oxidase gene, showed a 3.5-fold increase in regeneration frequency, demonstrating the inhibitory role of ethylene in melon regeneration (Ben Amor et al. 1998).

Environmental conditions affect shoot induction. Optimum light intensity and temperature are required for extensive bud induction from cotyledon explants (Niedz et al. 1989). However, bud formation from hypocotyls attached to a fragment of cotyledon appeared to be independent of light and could occur with the same efficiency in the dark, contrary to regeneration from cotyledonary explants (Curuk et al. 2003).

## 4.2 Somatic Embryogenesis

In general, regeneration from callus and other tissues of melon and cucurbits via somatic embryogenesis has not been very successful so far. This is due mainly to difficulties in the development of embryos and endo-polyploidy. In addition, the process is highly genotype dependent (Oridate et al. 1992).

An efficient method for the production of diploid plants by somatic embryogenesis employing an optimized two-step protocol has been described for a charentais type melon (cv. Védraçais) with a yield of 10 embryos per seed (Guis et al. 1997a). Using the same genotype, Akasaka-Kennedy et al. (2004) recently obtained a 10-fold increase in the yield (100 embryos per seed) by increasing the number of cuts to 20 per seed as compared to only 2 by Guis et al. (1997a), and by introducing an embryo induction phase in liquid medium of similar composition. The percentage of regenerated diploid plants was comparable in the two conditions (66–68%).

Auxins are required for the induction of somatic embryogenesis in melon (Tabei et al. 1991), with 2,4-dichlorophenoxyacetic acid (2,4-D) being generally the most efficient (Oridate and Oosawa 1986; Tabei et al. 1991). 2,4-D has often been used in combination with a cytokinin, generally BA (Akasaka-Kennedy et al. 2004). On some occasions, BA was replaced by thidiazuron in order to enhance embryo formation (Gray et al. 1993; Liborio Stipp et al. 2001). After the induction phase, somatic embryo maturation is usually achieved in hormone-free medium.

Light is also required for induction of embryos, although a transient period in the dark can be beneficial (Gray et al. 1993; Guis et al. 1997a). Based on the accumulation of some micro-nutrients in melon tissue cultures, Kintzios et al. (2004) suggested that regeneration from cotyledons by organogenesis could be improved by a  $\text{PO}_4^{3-}$ -enriched medium, while induction of embryogenesis could be promoted on  $\text{Mg}^{2+}$ -enriched medium. However, these suggestions remain to be tested.

### 4.3 Plant Regeneration from Protoplasts

Because of sexual incompatibility between *Cucumis* species, the possibility of overcoming fertilization barriers by somatic hybridization has been investigated. The methodology includes the isolation of protoplasts, followed by protoplast fusion and regeneration of whole plants. The regeneration of whole plants from protoplasts of melon has been reported previously (Tabei et al. 1987; Li et al. 1990; Bokelmann et al. 1991; Debeaujon and Branchard 1992). However, reports on high-frequency regeneration of normal plants are limited (Tabei et al. 1992). In general, regeneration rates remain low; abnormal shoots are frequently obtained and are unable to give whole mature plants (Jarl et al. 1995). Regeneration from protoplast fusion products has proved very difficult. Calli have been obtained after protoplast fusion between an albino mutant of melon and *C. myriocarpus* (Bordas et al. 1998) or *C. anguria longipes* (Dabauza et al. 1998). Molecular analysis revealed that the calli were hybrid, but no plants were regenerated. Although an attempt has been made to produce embryogenic protoplast-derived calli (Debeaujon and Branchard 1992), the efficiency in terms of plant regeneration is still low.

### 4.4 Hyperhydricity

Beside endo-ploidy, a physiological problem often encountered in cultured melon tissue is the development of a hyperhydric texture and glassy appearance of the tissues (named 'hyperhydricity', previously known as 'vitrification'). Leshem et al. (1988) considered that high cytokinin concentrations are the prime inducers of hyperhydricity in melon buds, but many other factors are also probably involved, such as wounding, soft culture medium, high ionic strength, high nitrogen under unbalanced hormonal composition and a highly humid atmosphere (Kevers et al. 2004).

### 4.5 Genetic Control

The regeneration response of melon cultivars is highly genotype dependent. High regeneration rates have been observed in some genotypes from hypocotyl callus (Kathal et al. 1986), somatic embryos (Tabei et al. 1991) and leaves (Yadav et al. 1996), while poor regeneration has been observed in others by organogenesis from cotyledons or leaves and by embryogenesis (Gaba et al. 1999; Liborio-Stipp et al. 2001). It is generally considered that *reticulatus* genotypes give better regeneration rates via embryogenesis (Oridate et al. 1992; Gray et al. 1993), while *inodorus* cultivars give greater organogenesis rates (Ficcadenti and Rotino 1995). Genetic studies using a segregating population of melon have suggested that two partially dominant loci are involved in the determination of competence to regenerate by organogenesis (Molina and Nuez 1996) and that their competence is sexually transmissible (Molina and Nuez 1997). An in-

bred melon genotype named BU-21/3 has been generated that shows superior competence for regeneration via organogenesis from cotyledons and hence for transformation compared to previously evaluated genotypes, including American cantaloupes and charentais types (Galperin et al. 2003a). Crossing the BU-21/3 line with the two *reticulatus* cultivars, which were virtually incompetent for organogenesis, showed that the character of high competence for regeneration is controlled by a single dominant locus (Galperin et al. 2003b).

## 5 Haploidization, Triploids and Somatic Hybridisation

There has been no major innovation in the production of haploid plants or in somatic hybridization in melon since the last review on melon biotechnology (Guis et al. 1998). Induction of in situ haploid parthenogenesis by irradiated pollen is still the method of choice (Sauton and Dumas de Vaulx 1987). Some improvements have been proposed. A preculture of seeds in liquid medium before placing the embryos on a semi-solid culture medium significantly improved the number of haploid plants (Lotfi et al. 2003). Immersion of in vitro cuttings into a colchicine solution has been proposed by Sauton (1988) in order to double the chromosome number so as to obtain double-haploid plants. An alternative method, by immersing shoot tips of haploid plants grown under glasshouse conditions, is more efficient (Yetisir and Sari 2003).

The different steps in the procedure, i.e., the frequency of haploid plant production and the efficiency of doubling the chromosome number, are genotype dependent, but there has been no study on their genetic control. Resistance or susceptibility to powdery mildew is expressed and selection can be done at the haploid level (Kuzuya et al. 2003).

Triploid seedless watermelons have become more and more popular, but nothing similar has been obtained in melon. A fertile allotetraploid has been obtained by sexual hybridization between cucumber (*C. sativus*) and *Cucumis hystris* Chakravarty and named *C. hytivus* Chen and Kirkbride (Chen et al. 2003a). Allotriploids were obtained between *C. hytivus* and *C. sativus* (Chen et al. 2003b). Nothing similar has been obtained between melon and other species of *Cucumis*.

## 6 Methods of Genetic Transformation

Details of the first successful transformation of melon were published in the early 1990s (Fang and Grumet 1990; Dong et al. 1991). Subsequently, transgenic melon plants have been generated through the introduction of genes for virus resistance, halotolerance, herbicide resistance and long fruit shelf-life (Table 4). Melon transformation has been most commonly performed through

*Agrobacterium tumefaciens* using mainly cotyledon explants. Some *Agrobacterium* strains seem more efficient than others (Guis et al. 1998), and inhibition of ethylene production by aminovinylglycine improves the transformation efficiency (Ezura et al. 2000). Recently, a transformation method by wounding melon explants with carborundum, prior to *Agrobacterium* inoculation, was developed (Curuk et al. 2005). This method enabled the successful transformation of recalcitrant varieties at a rate reaching 5% (percent of explants that give rise to transformed plants). The melon genotype 'BU-21/3', with a superior competence for regeneration (Galperin et al. 2003a), also gave a very high rate of genetic transformation of 0.4–1.5 transgenic shoots per explant.

Thanks to important progress in the efficiency of embryogenic regeneration, an *Agrobacterium*-mediated transformation protocol using a liquid medium culture step was devised, in which a rate of transformation exceeding 2.3% has been achieved (Akasaka-Kennedy et al. 2004). Furthermore, the protocol was reported to strongly reduce the number of false transformants ('escapes') at a kanamycin concentration of 25 mg l<sup>-1</sup>. This protocol represents significant progress towards the routine transformation of melon, as it seems to be efficient for a wide range of melon genotypes (Ezura, personal communication).

Particle gun transformation has also been used that gives a transformation efficiency sometimes similar to that of *Agrobacterium* (Gonsalves et al. 1994; Gray et al. 1995). The most frequently used selectable marker gene is *nptII* conferring resistance to kanamycin. However, escapes to kanamycin are generally high in melon, so that authors have tried to optimize the concentration of antibiotic during the different phases of regeneration (Guis et al. 1998).

In conclusion, despite significant progress made in the methods, melon transformation still has poor efficiency (with transformation rates of around 2–7%, except for specific genotypes), contrary to other horticultural species such as tomato, for which genotype-independent and routine protocols are available that give transformation rates as high as 10–15% (Frary and Van Eck 2004).

Because selectable marker genes conferring resistance to antibiotics are considered as carrying a number of health and environmental risks, methods have been developed that can eliminate or avoid such marker genes. A procedure based on the use of an *A. tumefaciens* mutant has been applied to melon (Mihalka et al. 2003). This mutant is deficient in the auxin biosynthesis genes and confers an enhanced level of cytokinin expression in transformed plant cells due to the expression of the T-DNA isopentenyl transferase (*ipt*) gene. Using such a mutant, transgenic shoots could be selected in the absence not only of exogenous growth regulators, such as cytokinins, but also of selective agents such as antibiotics or herbicides. The expression of the *ipt* gene normally leads to transgenic shoots with an abnormal morphology named 'extreme shooty phenotype'. However, some shoots showed a normal phenotype due to the recurrent but still unexplained absence of the *ipt* gene in some regenerants. In these cases, when a binary vector containing a gene of interest was introduced into the *A. tumefaciens* mutant, marker-free regenerants could be selected by screening for the normal phenotype.

Table 4. Genes of horticultural interest introduced into *Cucumis melo* by genetic transformation

Gene	Transformation method	Regeneration method and type of explant	Agricultural traits	Reference
Cucumber mosaic virus (CMV) coat protein gene	<i>A. tumefaciens</i>	Organogenesis Cotyledon	CMV resistance	Yoshioka et al. (1993)
Zucchini yellow mosaic virus (ZYMV) coat protein gene	<i>A. tumefaciens</i>	Organogenesis Cotyledon	ZYMV resistance	Fang and Grumet (1993)
CMV-WL coat protein gene	<i>A. tumefaciens</i> and biolistics	Organogenesis Cotyledon	CMV resistance	Gonsalves et al. (1994)
ZYMV, watermelon mosaic virus (WMV), CMV coat protein genes	<i>A. tumefaciens</i>	Organogenesis Leaf disks	ZYMV, WMV, CMV resistance	Clough and Hamm (1995)
Antisense ACC oxidase gene (ethylene biosynthesis)	<i>A. tumefaciens</i>	Organogenesis Cotyledon	Slow ripening	Ayub et al. (1996)
ZYMV, WMV, CMV coat protein genes	<i>A. tumefaciens</i>	Organogenesis Cotyledon	ZYMV, WMV, CMV resistance	Fuchs et al. (1997)
Polyribozyme against CMV	<i>A. tumefaciens</i>	Organogenesis Cotyledon	CMV resistance	Plages (1997)
HAL1 yeast gene	<i>A. tumefaciens</i>	Organogenesis Leaf and Cotyledon	Salt resistance	Bordas et al. (1997)
S-adenosylmethionine hydrolase gene	<i>A. tumefaciens</i>	Not mentioned	Slow ripening	Serrano et al. (1999)
Bar gene	Potyvirus-vector inoculation	Direct inoculation on whole plant	Herbicide resistance	Glendennen et al. (1999)
Polyribozyme genes	<i>A. tumefaciens</i>	Organogenesis Cotyledon	WMV2, ZYMV resistance	Shiboleth et al. (2001)
Photorespiratory eR genes	<i>A. tumefaciens</i>	Organogenesis Cotyledon	Downy mildew resistance	Huttner et al. (2001)
Apple ACC oxidase (ethylene biosynthesis)	<i>A. tumefaciens</i>	Organogenesis Cotyledon	Slow ripening	Taler et al. (2004)
Petunia ACC synthase	<i>A. tumefaciens</i>	Organogenesis Cotyledon	Earlier floral development and fruit set	Silva et al. (2004)
				Papadopoulos et al. (2005)

Transfection of whole melon plants with foreign genes, without going through the regeneration process, has been achieved using the AGII potyvirus-based vector system (Arazi et al. 2001). The AGII vector has been created from an attenuated strain of ZYMV which has been rendered unable to elicit phenotypic impairment of plant development and to be transmitted by aphids.

## 7 Genetic Transformation for Disease Resistance

Besides the use of natural genetic resistance present within the *Cucumis melo* germplasm in breeding programmes, the production of transgenic plants resistant to pests and diseases is an alternative method of crop improvement. In melon, the pathogen-derived resistance approach was mainly investigated as a mean to develop virus-resistant plants. Progress made with the production of multiple virus-resistant transgenic cucurbits, including melon, have been reviewed recently by Gaba et al. (2004). Transgenic melons have been produced over-expressing the intact coat protein (CP) gene of CMV, ZYMV and watermelon mosaic virus (WMV). CP-mediated protection was shown to give apparent immunity to ZYMV infection (Fang and Grumet 1993), but, in most cases, it only delayed the appearance of viral disease symptoms (Yoshioka et al. 1993; Gonsalves et al. 1994; Clough and Hamm 1995). A partial resistance under field conditions was also provided by the association of the three CP genes (CMV, ZYMV and WMV), leading to significantly reduced infection by each virus and a reduced incidence of mixed infections (Fuchs et al. 1997). The level of the CP-mediated resistance depended on the level of CP gene product in the transgenic plant. Thus, plants homozygous for the transgene exhibited a higher level of resistance under field conditions than hemizygous plants (Fuchs et al. 1997). Combinations of as many as five viral CP genes (ZYMV, CMV, WMV, SqMV and PRSV-W) were transferred in melons and assessed under field conditions (Gaba et al. 2004), but the efficiency of these transgenes towards multiple virus infection has not been documented. The expression of the conserved central 'core' of the ZYMV CP or the expression of the antisense gene of the ZYMV CP gave only limited protection against ZYMV (Fang and Grumet 1993).

In melon, ribozyme-mediated resistance has been observed against CMV (Plages 1997), ZYMV and WMV (Huttner et al. 2001). This type of resistance is based on the ability of ribozymes, small RNA molecules, to cleave viral RNA with high specificity. Melon plants containing polyribozyme targeted against WMV were found to be highly resistant to WMV under field conditions (Huttner et al. 2001).

To our knowledge, there have been no reports on the comparison of agronomic performance, under identical conditions, of transgenic melons and conventional resistant (even partially resistant) genotypes. Also, the behaviour of plants carrying a transgene and a natural resistant background has never been

tested. Sources of natural resistance to CMV and ZYMV have been reported over several years in melon, and have been incorporated into cultivars by breeding.

## 8 Genetic Transformation for Desirable Fruit Quality Traits and Post-Harvest Behaviour

Improvement of fruit quality and post-harvest behaviour through biotechnology has been attempted, so far, only in cantaloupe melons of the *cantalupensis* or *reticulatus* type. The ripening process of these melons is of the climacteric type and is controlled by the hormone ethylene (Lelièvre et al. 1997). Under these conditions, ethylene biosynthetic genes have been the first obvious target genes to be used for controlling the ripening process. Three ACC synthase (ACS) and three ACC oxidase (ACO) genes have been isolated and characterized in melon (Yamamoto et al. 1995; Lasserre et al. 1996). An antisense construct of an ACO cDNA driven by the 35S promoter has been used for generating transgenic cantaloupe charentais melons (Ayub et al. 1996). A line of the antisense ACO melon showed a reduction in ethylene production by more than 99.5%, which resulted in an inhibition of rind yellowing, fungal attack and peduncle detachment (Figs. 2, 3), as well as a slowing down of flesh softening, inhibition of climacteric respiration (Fig. 3; Guis et al. 1997b), production of aroma volatiles (Bauchot et al. 1998) and genes involved in the production of ester volatiles (El-Sharkawy et al. 2005; Manríquez et al. 2006). Low concentrations of ethylene ( $2.5\text{--}5\ \mu\text{L L}^{-1}$ ) were able to restore the original ripening phenotype (Flores et al. 2001). Some ripening pathways, such as coloration of the flesh and accumulation of sugars (Fig. 3) and organic acids, were not affected by ethylene suppression. The expression of an antisense ACO of apple in a charentais melon also resulted in a very strong reduction in ethylene production and led to similar conclusions in terms of ethylene-regulated and ethylene-dependent pathways (Silva et al. 2004). In addition to the ripening phenotype, the antisense ACO melons have shown increased tolerance to low temperature disorders during and after storage at  $2\ ^\circ\text{C}$  compared with wild-type plants (Ben Amor et al. 1999). Such tolerance was even improved under modified atmosphere packaging (Flores et al. 2004). Chilling tolerance was correlated with a reduced accumulation of ethanol and acetaldehyde and a higher activity of enzymes capable of removing activated oxygen species, such as catalase and superoxide dismutase (Ben Amor et al. 1999).

Reducing ethylene production has also been achieved by expressing the T3 bacteriophage S-adenosylmethionine hydrolase (SAMase) under the control of a chimeric fruit-specific promoter in American-type cantaloupes (Clendennen et al. 1999). By catalyzing the degradation of SAM, a precursor to ethylene synthesis, melons expressing SAMase produced 75% less ethylene than control



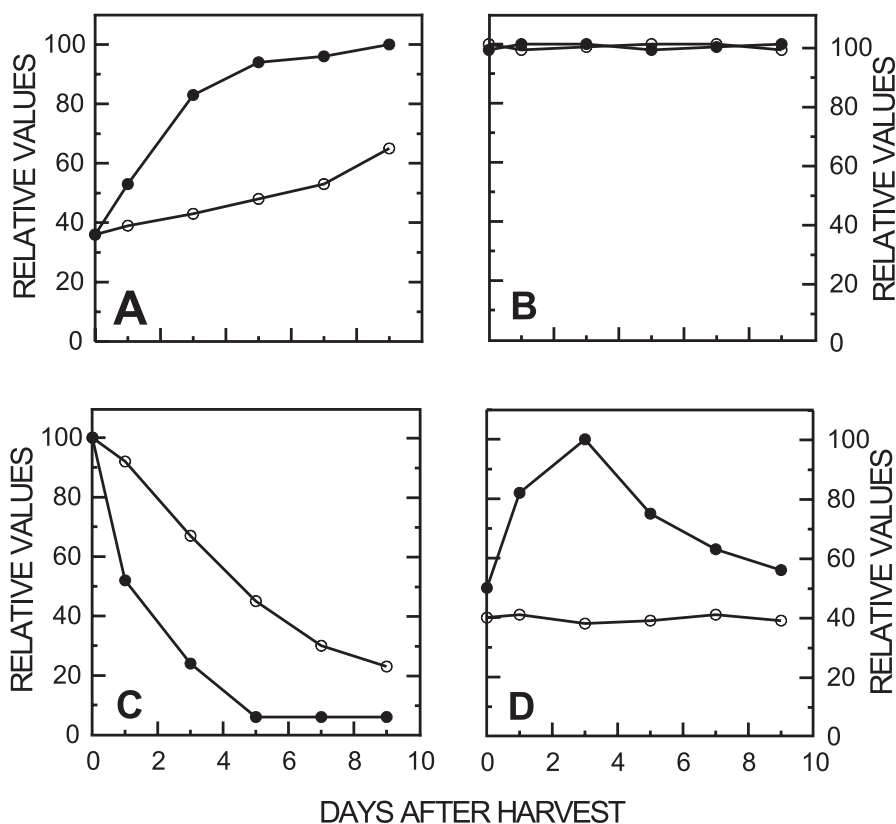


**Fig. 2.** Wild-type (WT, *left*) and ACO antisense melon fruit (AS, *right*) harvested 32 days after pollination and held at 20 °C for 10 days. WT fruit has turned yellow and shows fungal attack, while AS fruit remains green without external signs of senescence. Bar 2 cm

fruit. However, the inhibition of ethylene production was not enough to change significantly the ripening and post-harvest behaviour. One of the very few effects of the transgene was a delay in the formation of the abscission zone which frequently allowed greater sugar accumulation on the vine.

These two examples indicate that inhibition of cantaloupe melon ripening requires strong inhibition of ethylene production due to the high sensitivity of the ripening pathways to low concentrations of ethylene (Flores et al. 2001). In these conditions two strategies could be developed at the practical level: (1) either inhibit totally ethylene production and then ripen the fruit after harvest by treating with ethylene, or (2) reduce the production of ethylene at a level capable of slowing down, but not blocking, the ripening process. It is reasonable to establish at 2–3  $\mu\text{L L}^{-1}$  the internal ethylene concentrations required in such a strategy for cantaloupe melons (Flores et al. 2001).

Genes involved in the ethylene perception and transduction pathway have been characterized in *Arabidopsis* (Nehring and Ecker 2004) and in fruit, mainly tomato (Klee and Clark 2005). The mutated version of the *Arabidopsis* ethylene receptor *etr1-1* has been transferred into heterologous plants (tomato, petunia and carnation) to confer ethylene insensitivity on flowers and fruit (Wilkinson et al. 1997; Bovy et al. 1999). In melon, two cDNA homologues of the *Arabidopsis* ethylene receptor genes, *Cm-ETR1* and *Cm-ERS1*, have been isolated that show differential expression during fruit development and ripening (Sato-Nara et al. 1999). A mutated version of the *Cm-ETR1* receptor gene of melon is capable of conferring reduced ethylene sensitivity in a heterologous plant, *Nemesia strumosa* (Cui et al. 2004). Reducing ethylene sensitivity in melon by expressing the mutated *Arabidopsis* ethylene receptor, ETR1-1



**Fig. 3.** Relative changes in ripening parameters of wild-type (*solid symbols*) and antisense ACC oxidase (*open symbols*) cantaloupe charentais melon held at 20–22 °C after harvest. **A** Colour of the rind measured in the  $L^*a^*b^*$  system by a Minolta chromameter as the  $b$  chromatic value corresponding to the intensity of yellow colour (100 corresponds to a  $b$  value of 35); **B** soluble solids (100 = 12°Brix) or carotenoid content of the pulp (100 = 18 mg kg<sup>-1</sup>); **C** firmness of the pulp (100 = 4.8 kg cm<sup>-2</sup>); **D** respiration (100 = 2.2 mmol O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>). Adapted from Guis et al. (1997b), Flores et al. (2001) and Bower et al. (2002)

(Little et al. 2006), demonstrates that ethylene perception is required for carpel development.

Improving specific aspects of fruit quality can also be achieved by altering the expression of ripening genes involved in the development of specific attributes. Many genes putatively involved in cell-wall polymer disassembly have been isolated in tomato, but their specific role in fruit softening is not fully elucidated (Rose and Bennett 1999). In addition, enzymatic and non-enzymatic mechanisms contribute to cell wall disassembly (Rose et al. 2004). In melon, three polygalacturonase genes have been isolated whose expression pattern suggests a role in pectin degradation during fruit ripening (Hadfield et al. 1998). These genes could serve as a target for reducing softening of the flesh.

However, it is probable that, similar to reduction of softening in tomato, this may involve the slowing down of several cell wall-degrading enzymes or cell wall proteins. Genes involved in the production of aroma volatiles have been isolated in charentais melon that could also be employed for biotechnological purposes (Yahyaoui et al. 2002). Another interesting gene of considerable potential in melon biotechnology is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). This enzyme participates in the synthesis of mevalonate, a precursor of a number of hormones (abscissic acid, cytokinins, gibberellins and brassinosteroids) and is potentially involved in cell division of the melon pericarp (Kobayashi et al. 2003a). Expression of the HMGR gene in tomato has resulted in increased fruit size (Kobayashi et al. 2003b), but so far no data are available on over-expression in melon.

In all these studies, the availability of fruit-specific promoters would be of interest for directing gene expression specifically in fruit and not in other plant parts where expression of the transgene is not sought or is not desirable. The promoter of the cucumisin gene of melon, capable of driving gene expression specifically in the fruit, has been characterized (Yamagata et al. 2002). The TGTCACA motif appears to be a cis-regulatory enhancer element necessary for fruit-specific expression (Yamagata et al. 2002). A chimaeric fruit-specific promoter derived from a modification of the actin gene promoter of melon has been patented (Clendennen and Kellogg 2002).

## 9 Genetic Transformation for Resistance to Salt and Herbicides

The adaptation of plants to environmental abiotic stresses, such as drought and salinity, involves the activation of a wide range of genes whose individual functions are not well understood. In fact, most of these genes are not specific to one type of stress. A few studies have been carried out, mostly on model plants such as *Arabidopsis* and tobacco and, to a lesser extent, tomato. These consisted in overproduction of osmolytes, the expression of enzymes capable of reducing reactive oxygen species, the expression of proteins involved in  $\text{Na}^+$  and  $\text{K}^+$  transport, and overproduction of stress-inducible transcription factors (Apse and Blumwald 2002; Borsani et al. 2003). In melon, saline stress is of interest, since it results in an alteration of the ion balance of the cell. Expression of a yeast salt-tolerance gene encoding a water-soluble protein HAL1 involved in  $\text{K}^+$  and  $\text{Na}^+$  transport has been achieved in melon (Bordas et al. 1997). It resulted in some degree of sexually transmissible salt tolerance during *in vitro* tests (Serrano et al. 1999). Evaluation in the field has not yet been attempted for melon, but tests have been carried out with tomato in which enhanced tolerance has indeed been confirmed under glasshouse conditions.

Cucurbits, including melon, are sensitive to a wide range of herbicides, so that no selective herbicide can be applied post weed emergence. Engineering of cucurbits for resistance to glufosinate ammonium-based herbicides has been

performed by expressing a *bar* gene (phosphinothricin acetyl transferase) via infection by an attenuated potyvirus vector, AGII, as described in Sect. 6 (Arazi et al. 2001). Field spraying of ammonium glufosinate on the foliage of 2-week-old melons and other cucurbits inoculated with the AGII-*bar* construct allowed the elimination of weeds without inducing deleterious symptoms by the plants, even at the highest doses (Shiboleth et al. 2001).

## 10 Conclusions

The application of genetic engineering to plants is relatively recent (Herrera-Estrella et al. 1983). One of the first transgenic crops allowed for commercial release in the USA was a summer squash, Freedom II (Arce-Ochoa et al. 1995; Wilkinson 1997). In France, a CMV-resistant melon based on ribozyme technology has been registered but not commercialized (Plages 1997). Transgenic horticultural products represent less than 0.5 million ha, as compared to over 80 million ha for major agricultural crops such as soybean, corn, cotton and canola ([www.isaaa.org](http://www.isaaa.org)). In the specific case of melon, the number of field tests that have been undertaken is considerably low. From the data reported by Gaba et al. (2004), the number of field tests of virus-resistant transgenic melons represents 1.3% of the total number of field test applications in the USA, most of them for resistance to several viruses. In the European Union (Spain, Italy and France), there were six field test applications for transgenic melons up until April 2001 (Gaba et al. 2004). Public acceptance is a very strong limitation for the commercial release of transgenic melon varieties. Even in the USA, where two squash varieties have been permitted for sale, transgenic melons have not been released onto the market. Public reluctance is probably greater for fresh fruit and vegetables than for industrial processed crops. Within this context, research efforts are being made to render transgenic fresh fruit acceptable to the consumer.

Potential environmental risks associated with genetically engineered crops have been investigated by different approaches. One of the most widely discussed issues involves the potential for engineered genes to spread via pollen into populations of crop wild relatives or into non-transgenic plants. Hokanson et al. (1997) found that pollen dispersal of a native gene and a transgene of melon under field conditions was nearly identical. An additional concern is that pathogen-derived transgenes could recombine with wild pathogens, creating a new chimerical virus with unknown properties. Thus, Fuchs et al. (1998) explored the potential of transgenic plants expressing CP genes for triggering changes in virus-vector specificity. They showed that a transgenic melon containing the CP of an aphid-transmissible strain of CMV (WL) grown under field conditions has been unable to assist the spread of the aphid non-transmissible strain C of CMV through heterologous encapsidation or recombination. The influence of virus-resistant transgenic melons on the soil microflora and their

potential for producing specific products influencing the environment and other plants has been investigated by Tabei et al. (1994). However, transgenic plants were shown to exert negligible effect on the environment.

Agronomic traits of interest to the consumer, such as nutritional and sensory quality or resistance to biotic and abiotic stresses, are being increasingly researched. Technologies for the expression of transgenes become more and more sophisticated, with the use of organ-specific promoters and the absence of antibiotic resistance marker genes. These new technologies are already available for melon. They should assist in transgenic plants gaining public acceptability.

Considerable progress has been made in the genetic mapping of the agronomic characteristics of melon that has paved the way towards the introgression of new characters from wild and exotic accessions. Biotechnology is a method of choice not only for the functional identification of genes that have been mapped and isolated through positional cloning, but also for transferring specific characters into elite parent genotypes. It is predicted that genetics and biotechnology will become more and more complementary in striving for the improvement of agronomic and quality traits.

**Acknowledgements.** We thank the Midi-Pyrénées Regional Council (grants 01008920 and 03001146), whose financial support has made possible our own research on melon biotechnology and the writing of this chapter.

## References

- Akasaka-Kennedy Y, Tomita K, Ezura H (2004) Efficient plant regeneration and *Agrobacterium*-mediated transformation via somatic embryogenesis in melon (*Cucumis melo* L.). *Plant Sci* 166:763–769
- Akashi Y, Fukuda N, Wako T, Masuda M, Kato K (2002) Genetic variation and phylogenetic relationships in east and south Asian melons, *Cucumis melo* L., based on analysis of five isozymes. *Euphytica* 125:385–396
- Apse MP, Blumwald E (2002) Engineering salt tolerance in plants. *Curr Opin Biotechnol* 13:146–150
- Arazi T, Slutzky G, Shibolet Y, Wang Y, Rubinstein M, Barak M, Yang J, Gal-On A (2001) Engineering zucchini mosaic potyvirus as a non-pathogenic vector for expression of heterologous proteins in cucurbits. *J Biotechnol* 87:67–82
- Arce-Ochoa JP, Dainello F, Pike LM, Drews D (1995) Field performance comparison of two transgenic summer squash hybrids to their parental hybrid line. *HortScience* 30:492–493
- Arumanagathan K, Earle ED (1991) Nuclear DNA content of some important plant species. *Plant Mol Biol Rep* 9:208–218
- Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latché A, Bouzayen M, Pech JC (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. *Nat Biotechnol* 14:862–866
- Baba E, Zarka V, Deak T, Pedryc A, Velich I, Bisztray GD (2002) Molecular diversity of Hungarian melon varieties revealed by RAPD markers. *Int J Hort Sci* 8:11–13

- Bauchot AD, Mottram DS, Dodson AT, John P (1998) Effect of aminocyclopropane-1-carboxylic acid oxidase antisense gene on the formation of volatile esters in cantaloupe charentais melon (cv. Védraçais). *J Agric Food Chem* 46:4787–4792
- Baudracco-Arnas S, Pitrat M (1996) A genetic map of melon (*Cucumis melo* L.) with RFLP, RAPD, isozyme, disease resistance and morphological markers. *Theor Appl Genet* 93:57–64
- Ben Amor M, Guis M, Latché A, Bouzayen M, Pech JC, Roustan JP (1998) Expression of an antisense 1-aminocyclopropane-1-carboxylate oxidase gene stimulates shoot regeneration in *Cucumis melo*. *Plant Cell Rep* 17:586–589
- Ben Amor M, Flores B, Latché A, Bouzayen M, Pech JC, Romojaro F (1999) Inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevents chilling injury in charentais cantaloupe melons. *Plant Cell Environ* 22:1579–1586
- Bernadac A, Latché A, Roustan JP, Bouzayen M, Pech JC (2002) Cucurbits, pepper, eggplant, legumes and other vegetables. In: Valpuesta V (ed) *Fruit and vegetable biotechnology*. CRC Press, Woodhead, Cambridge, pp 250–293
- Bokelmann GS, Jarl CI, Kool AJ (1991) Plant regeneration of protoplasts from different cultivars of melon (*Cucumis melo*). *Cucurbit Gene Coop Rep* 14:78–80
- Bordas M, Montesinos C, Dabauza M, Salvador A, Roig AL, Serrano R, Moreno V (1997) Transfer of the yeast salt tolerance gene HAL1 to *Cucumis melo* L. cultivars and in vitro evaluation of salt tolerance. *Transgenic Res* 6:41–50
- Bordas M, Gonzales-Candelas L, Dabauza M, Ramon D, Moreno V (1998) Somatic hybridization between an albino *Cucumis melo* L. mutant and *Cucumis myriocarpus* Naud. *Plant Sci* 132:179–190
- Borsani O, Valpuesta V, Botella MA (2003) Developing salt tolerant plants in a new century: a molecular biology approach. *Plant Cell Tissue Organ Cult* 73:101–115
- Bovy AG, Angenent GC, Dons HJM, van Altvorst AC (1999) Heterologous expression of the *Arabidopsis etr1-1* allele inhibits the senescence of carnation flowers. *Mol Breed* 5:301–308
- Bower J, Holford P, Latché A, Pech JC (2002) Culture conditions and detachment of the fruit influence the effect of ethylene on the climacteric respiration of melon. *Postharvest Biol Technol* 26:135–146
- Brotman Y, Silberstein L, Kovalski I, Périn C, Dogimont C, Pitrat M, Klinger J, Thompson G, Perl-Treves R (2002) Resistance gene homologues in melon are linked to genetic loci conferring disease and pest resistance. *Theor Appl Genet* 104:1055–1063
- Brotman Y, Kovalski I, Dogimont C, Pitrat M, Portnoy V, Katzir N, Perl-Treves R (2005) Molecular markers linked to papaya ring spot virus resistance and *Fusarium* race 2 resistance in melon. *Theor Appl Genet* 110:337–345
- Chen J, Staub J, Qian C, Jiang J, Luo X, Zhuang F (2003a) Reproduction and cytogenetic characterization of interspecific hybrids derived from *Cucumis hystris* Chakr. × *Cucumis sativus* L. *Theor Appl Genet* 106:688–695
- Chen JF, Luo XD, Staub JE, Jahn MM, Qian CT, Zhuang FY, Ren G (2003b) An allotriploid derived from amphidiploid × diploid mating in *Cucumis* I: production, micropropagation and verification. *Euphytica* 131:235–241
- Clendennen SK, Kellogg JA (2002) Melon promoters for expression of transgenes in plants. US Patent 20020133850
- Clendennen SK, Kellogg JA, Wolff KA, Matsumura W, Peters S, Vanwinkle JE, Copess B, Pieper W, Kramer MG (1999) Genetic engineering of cantaloupe to reduce ethylene biosynthesis and control ripening. In: Kanellis AK, Chang C, Klee H, Bleecker AB, Pech JC, Grierson D (eds) *Biology and biotechnology of the plant hormone ethylene*. Kluwer, Dordrecht, pp 371–379
- Clough GH, Hamm PB (1995) Coat protein transgenic resistance to watermelon mosaic and zucchini yellows mosaic virus in squash and cantaloupe. *Plant Dis* 79:1107–1109
- Cui ML, Takada K, Ma B, Ezura H (2004) Over-expression of a mutated melon ethylene receptor gene CmETR1/H69A confers reduced ethylene sensitivity in a heterologous plant, *Nemesia strumosa*. *Plant Sci* 167:253–258

- Curuk S, Elman C, Schlarman E, Sagee O, Shomer I, Cetiner S, Gray DJ, Gaba V (2002) A novel pathway for rapid shoot regeneration from the proximal zone of the hypocotyl of melon (*Cucumis melo* L.). In *Vitro Cell Dev Biol-Plant* 38:260–267
- Curuk S, Ananthakrishnan G, Singer S, Xia X, Elman C, Nestel D, Cetiner S, Gaba V (2003) Regeneration in vitro from the hypocotyl of *Cucumis* species produces almost exclusively diploid shoots and does not require light. *HortScience* 38:105–109
- Curuk S, Cetiner S, Elman C, Xia X, Wang Y, Yeheskel A, Zilberstein L, Perl-Treves R, Watad AA, Gaba V (2005) Transformation of recalcitrant melon (*Cucumis melo* L.) cultivars is facilitated by wounding with carborundum. *Eng Life Sci* 5:169–177
- Dabauza M, Gonzales-Candelas L, Bordas M, Roig LA, Ramon D, Moreno V (1998) Regeneration and characterization of *Cucumis melo* L. (+) *Cucumis anguria* L. var. *longipes* (Hook.fil.) Meeuse somatic hybrids. *Plant Cell Tissue Organ Cult* 52:123–131
- Dane F (1983) Cucurbit. In: Tanksley SD, Orton TJ (eds) *Isozymes in plant genetics and breeding*, Part B. Elsevier, Amsterdam, pp 369–390
- Dane F (1991) Cytogenetics of the genus *Cucumis*. In: Tsuchiya T, Gupta PK (eds) *Chromosome engineering in plants: genetics, breeding, evolution*, part B. Elsevier, Amsterdam, pp 201–214
- Dangl JL, Jones JD (2001) Plant pathogens and integrated defense responses to infection. *Nature* 411:826–833
- Danin-Poleg Y, Reis N, Baudracco-Arnas S, Pitrat M, Staub JE, Oliver M, Arús P, de Vicente CM, Katzir N (2000) Simple sequence repeats in *Cucumis* mapping and map merging. *Genome* 43:963–974
- Danin-Poleg Y, Tadmor Y, Tzuri G, Reis N, Hirschberg J, Katzir N (2002) Construction of a genetic map of melon with molecular markers and horticultural traits, and localization of genes associated with ZYMV resistance. *Euphytica* 125:373–384
- Debeaujon I, Branchard M (1992) Induction of somatic embryogenesis and caulogenesis from cotyledon and leaf protoplast-derived colonies of melon (*Cucumis melo* L.). *Plant Cell Rep* 12:37–40
- Decker-Walters DS, Chung SM, Staub JE, Quemada HD, López-Sesé AI (2002) The origin and genetic affinities of wild populations of melon (*Cucumis melo*, Cucurbitaceae) in North America. *Plant Syst Evol* 233:183–197
- Dogimont C, Lecomte L, Périn C, Thabuis A, Lecoq H, Pitrat M (2000) Identification of QTLs contributing to resistance to different strains of cucumber mosaic cucumovirus in melon. In: Katzir N, Paris H (eds) *Proc Cucurbitaceae 2000, 7th Eucarpia Meeting on Cucurbit Genetics and Breeding*, Ma'ale Hahamisha, pp 391–398
- Dong JZ, Yang MZ, Jia SR, Chua NH (1991) Transformation of melon (*Cucumis melo* L.) and expression from cauliflower mosaic virus 35S promoter in transgenic melon plants. *Bio/Technology* 9:858–863
- Eduardo I, Arus P, Monforte AJ (2005) Development of a genomic library of near isogenic lines (NILs) in melon (*Cucumis melo* L.) from the exotic accession PI161375. *Theor Appl Genet* 112:139–148
- El-Sharkawy I, Manríquez D, Flores FB, Regad F, Bouzayen M, Latché A, Pech JC (2005) Functional characterization of a melon alcohol acyl-transferase gene family involved in the biosynthesis of ester volatiles. Identification of the crucial role of athreonine residue for enzyme activity. *Plant Mol Biol* 59:345–362
- Esquinas Alcazar JT (1981) Allozyme variation and relationships among Spanish land races of *Cucumis melo* L. *Kulturpflanze* 29:337–352
- Ezura H, Yuhashi KI, Yasuta T, Minamisawa K (2000) Effect of ethylene on *Agrobacterium tumefaciens*-mediated gene transfer to melon. *Plant Breed* 119:75–79
- Fang G, Grumet R (1990) *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. *Plant Cell Rep* 9:160–164
- Fang GW, Grumet R (1993) Genetic-engineering of potyvirus resistance using constructs derived from the zucchini yellow mosaic-virus coat protein gene. *Mol Plant-Microbe Interact* 6:358–367



- Fanourakis NE (1988) Possibility of interspecific hybridization between *Cucumis metuliferus* N. and *Cucumis melo* L. by reciprocal grafting. In: Risser G, Pitrat M (eds) Proc Eucarpia Meeting 'Cucurbitaceae 88', Avignon, pp 181–186
- Ficcadenti N, Rotino GL (1995) Genotype and medium affect shoot regeneration of melon. *Plant Cell Tissue Organ Cult* 40:293–295
- Flores F, Ben Amor M, Jones B, Pech JC, Bouzayen M, Latché A, Romojaro F (2001) The use of ethylene-suppressed lines to assess differential sensitivity to ethylene of the various ripening pathways in cantaloupe melons. *Physiol Plant* 113:128–133
- Flores FB, Martínez-Madrid MC, Ben Amor M, Pech JC, Latché A, Romojaro F (2004) Modified atmosphere packaging confers chilling tolerance on ethylene-inhibited cantaloupe charentais melon fruit. *Eur Food Res Technol* 219:614–619
- Frary A, Van Eck J (2004) Organogenesis from transformed tomato explants. In: Pena L (ed) *Transgenic plants: methods and protocols*. Humana Press, Totowa, New Jersey, pp 141–150
- Fuchs M, McPerson JR, Tricoli D, McMaster JR, Deng RZ, Boeshore ML, Reynolds JF, Russell PF, Quemada HD, Gonsalves D (1997) Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field. *Mol Breed* 3:279–290
- Fuchs M, Klas FE, McPerson JR, Gonsalves D (1998) Transgenic melon and squash expressing coat protein genes of aphid-borne viruses do not assist the spread of an aphid non-transmissible strain of cucumber mosaic virus in the field. *Transgenic Res* 7:449–462
- Gaba V, Elman E, Watad AA, Gray DJ (1996) Ancymidol hastens in vitro bud development in melon. *HortScience* 31:1223–1224
- Gaba V, Schlarman E, Elman C, Sagee O, Watad AA, Gray DJ (1999) In vitro studies on the anatomy and morphology of bud regeneration in melon cotyledons. *In Vitro Cell Dev Biol-Plant* 35:1–7
- Gaba V, Zelcer A, Gal-On A (2004) Cucurbit biotechnology – the importance of virus resistance. *In Vitro Cell Dev Biol-Plant* 40:346–358
- Galperin M, Zelcer A, Kenigsbuch D (2003a) High competence for adventitious regeneration in the BU-21/3 melon genotype is controlled by a single dominant locus. *HortScience* 38:1167–1168
- Galperin M, Patlis L, Ovadia A, Wolf D, Zelcer A, Kenigsbuch D (2003b) A melon genotype with superior competence for regeneration and transformation. *Plant Breed* 122:66–69
- Gao ZH, Johansen E, Eyers S, Thomas CL, Ellis NTH, Maule AJ (2004) The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. *Plant J* 40:376–385
- Garcia E, Jamilena M, Alvarez JJ, Arnedo T, Oliver JL, Lozano R (1998) Genetic relationships among melon breeding lines revealed by RAPD markers and agronomic traits. *Theor Appl Genet* 96:878–885
- Garcia-Mas J, Oliver M, Gómez-Paniagua H, de Vicente MC (2000) Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. *Theor Appl Genet* 101:860–864
- Gonsalves C, Xue B, Yepes M, Fuchs M, Ling KS, Namba S, Chee P, Slightom JL, Gonsalves D (1994) Transferring cucumber mosaic virus-white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. *J Am Soc Hort Sci* 119:345–355
- Gonzalo MJ, Oliver M, Garcia-Mas J, Monfort A, Dolcet-Sanjuan R, Katzir N, Arus P, Monforte A (2005) Simple-sequence repeat markers used in merging linkage maps of melon (*Cucumis melo* L.). *Theor Appl Genet* 110:802–811
- Gray DJ, McColley DW, Compton ME (1993) High frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. *J Am Soc Hort Sci* 118:425–432
- Gray DJ, Hiebert E, Kelley KT, Compton ME, Gaba VP (1995) Comparison of methods to transform embryogenic cotyledons of melon. *HortScience* 30:788
- Guis M, Latché A, Pech JC, Roustan JP (1997a) An efficient method for production of diploid cantaloupe charentais melon (*Cucumis melo* L. var. *cantalupensis*) by somatic embryogenesis. *Sci Hort* 69:199–206

- Guis M, Botondi R, Ben Amor M, Ayub R, Bouzayen M, Pech JC, Latché A (1997b) Ripening-associated biochemical traits of cantaloupe charentais melons expressing an antisense ACC oxidase transgene. *J Am Soc Hort Sci* 122:748–751
- Guis M, Roustan JP, Dogimont C, Pitrat M, Pech JC (1998) Melon biotechnology. *Biotechnol Genet Eng Rev* 15:289–311
- Guis M, Ben Amor M, Latché A, Pech JC, Roustan JP (2000) A reliable system for the transformation of cantaloupe charentais melon (*Cucumis melo* L., var *cantaloupensis*) leading to a majority of diploid regenerants. *Sci Hort* 84:91–99
- Hadfield KA, Rose JKC, Debbie SY, Berka RM, Bennett AB (1998) Polygalacturonase gene expression in ripe melon fruit supports a role for polygalacturonase in ripening-associated pectin disassembly. *Plant Physiol* 117:363–373
- Havey MJ, McCreight JD, Rhodes B, Taurick G (1998) Differential transmission of the *Cucumis* organellar genomes. *Theor Appl Genet* 97:122–128
- Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* 303:209–213
- Hokanson SC, Hancock JF, Grumet R (1997) Direct comparison of pollen-mediated movement of native and engineered genes. *Euphytica* 96:397–403
- Huttner E, Tucker W, Vermeulen A, Ignart F, Sawyer B, Birch R (2001) Ribozyme genes protecting transgenic melon plants against potyviruses. *Curr Issues Mol Biol* 3:27–34
- Jarl CI, Bokelmann GS, De Hass JM (1995) Protoplast regeneration and fusion in *Cucumis*: melon × cucumber. *Plant Cell Tissue Organ Cult* 43:259–265
- Jeffrey C (1980) A review of the Cucurbitaceae. *Bot J Linn Soc* 81:233–247
- Joobeur T, King JJ, Nolin SJ, Thomas CE, Dean RA (2004) The fusarium wilt resistance locus *Fom-2* of melon contains a single resistance gene with complex features. *Plant J* 39:283–297
- Kato K, Akashi Y, Tanaka K, Wako T, Masuda M (2002) Genetic characterization of East and South Asian melons, *Cucumis melo*, by the analysis of molecular polymorphisms and morphological characters. *Acta Hort* 588:217–222
- Katzir N, Danin-Poleg Y, Tzuri G, Karchi Z, Lavi U, Cregan PB (1996) Length polymorphism and homologies of microsatellites in several Cucurbitaceae species. *Theor Appl Genet* 93:1282–1290
- Kevers C, Franck T, Strasser RJ, Dommes J, Gaspar T (2004) Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. *Plant Cell Tissue Organ Cult* 77:181–191
- Kathal R, Bhatnagar SP, Bhojwani SS (1986) Regeneration of shoots from hypocotyl callus of *Cucumis melo* cultivar ‘Pusa Sharbati’. *J Plant Physiol* 126:59–62
- Kathal R, Bhatnagar SP, Bhojwani SS (1988) Regeneration of plants from leaf explants of *Cucumis melo* cultivar ‘Pusa Sharbati’. *Plant Cell Rep* 7:449–451
- Kathal R, Bhatnagar SP, Bhojwani SS (1994) Plant regeneration from the callus derived from root explants of *Cucumis melo* L. cultivar ‘Pusa Sharbati’. *Plant Sci* 96:137–142
- Kintzios S, Stavropoulou E, Skamnelli S (2004) Accumulation of selected macronutrients and carbohydrates in melon tissue cultures: association with pathways of in vitro dedifferentiation and differentiation (organogenesis, somatic embryogenesis). *Plant Sci* 167:655–664
- Kirkbride JH (1993) Biosystematic monograph of the genus *Cucumis* (Cucurbitaceae). Parkway, Boone, North Carolina
- Klee HJ, Clark DG (2005) Ethylene signal transduction in fruits and flowers. In: Davies P (ed) *Plant hormones: biosynthesis, signal transduction, action!* Kluwer, Dordrecht, pp 369–390
- Kobayashi T, Kato-Emori S, Tomita K, Ezura H (2003a) Involvement of 3-hydroxyglutaryl coenzyme A reductase in cell division in the pericarp, which determines melon fruit size. *Acta Hort* 588:35–38
- Kobayashi T, Kato-Emori S, Tomita K, Ezura H (2003b) Transformation of tomato with the melon 3-hydroxy-3-methylglutaryl coenzyme A reductase leads to increase of fruit size. *Plant Biotechnol* 20:297–303
- Kuzuya M, Hosoya K, Yashiro K, Tomita K, Ezura H (2003) Powdery mildew (*Sphaerotheca fuliginea*) resistance in melon is selectable at the haploid level. *J Exp Bot* 54:1069–1074

- Lasserre R, Bouquin T, Hernandez J, Bull J, Pech JC, Balagué C (1996) Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Mol Gen Genet* 251:81–90
- Lebeda A, Kubalaková M, Kristkova E, Navrátilová B, Dolezal K, Dolezal J, Lysák M (1997) Morphological and physiological characteristics of plants issued from an interspecific hybridization of *Cucumis sativus* × *Cucumis melo*. In: Abak K, Büyükalaca S (eds) *Proc 1st Int Symp on Cucurbits*, Adana, pp 149–155
- Lelièvre JM, Latché A, Jones B, Bouzayen M, Pech JC (1997) Ethylene and fruit ripening. *Physiol Plant* 101:727–739
- Leshem B (1989) Polarity and responsive regions for regeneration in the cultured melon cotyledons. *J Plant Physiol* 135:237–239
- Leshem B, Shaley DP, Izhar S (1988) Cytokinin as an inducer of vitrification in melon. *Ann Bot* 61:255–260
- Li R, Sun Y, Zhang L, Li X (1990) Plant regeneration from cotyledon protoplasts of Xinjiang muskmelon. *Plant Cell Rep* 9:199–203
- Liborio-Stipp LC, Januzzi-Mendes BM, Stefano-Piedade SMD, Martinelli-Rodriguez AP (2001) In vitro morphogenesis of *Cucumis melo* var *inodorus*. *Plant Cell Tissue Organ Cult* 65:81–89
- Little HA, Hammar S, Grumet R. (2006) Studies of transgenic melon expressing the mutant ethylene receptor, ETR1-1, indicate that ethylene perception by stamen primordial is required for carpel development in melon flowers. *Cucurbitaceae proceedings 2006*. 17-21 September, Asheville, NC USA)
- Liu W, Song M, Liu F, Wang H (2002) Assessment of genetic diversity of melon (*Cucumis melo*) germplasm based on RAPD and ISSR. *J Agric Biotechnol* 10:231–236
- López-Sesé AI, Staub J, Katzir N, Gomez-Guillamon ML (2002) Estimation of between and within accession variation in selected Spanish melon germplasm using RAPD and SSR markers to assess strategies for large collection evaluation. *Euphytica* 127:41–51
- López-Sesé AI, Staub J, Gomez-Guillamon ML (2003) Genetic analysis of Spanish melon (*Cucumis melo*) germplasm using a standardized molecular-marker array and geographically diverse reference accessions. *Theor Appl Genet* 108:41–52
- Lotfi M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003) Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. *Plant Cell Rep* 21:1121–1128
- Luo M, Wang YH, Frisch D, Joobeur T, Wing R, Dean RA (2001) Melon BAC library construction using improved methods and identification of clones linked to the locus conferring resistance to melon *Fusarium* wilt (*Fom-2*). *Genome* 44:154–162
- Ma D, Guo Z, Zhang C, Gao S, Wang M (1994) Chromosome number and karyotype of melons (*Cucumis melo* L.). *Cucurbit Genet Coop Rep* 17:61–65
- Manríquez D, El-Sharkawy I, Flores FB, Regad F, Bouzayen M, Latché A, Pech JC (2006) Fruit-specific gene expression and biochemical characteristics of two highly divergent alcohol dehydrogenases of melon. *Plant Mol Biol* 61:675–685
- Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. *Annu Rev Plant Biol* 54:23–61
- McCreight JD, Staub JE, López Sesé A, Chung SM (2004) Isozyme variation in Indian and Chinese melon (*Cucumis melo* L.) germplasm collections. *J Am Soc Hort Sci* 129:811–818
- Meglic V, Horejsi TF, McCreight JD, Staub JE (1994) Genetic diversity and inheritance and linkage of isozyme loci in melon (*Cucumis melo* L.). *HortScience* 29:449
- Mihalka V, Balazs E, Nagy I (2003) Binary transformation systems based on “shooter” mutants of *Agrobacterium tumefaciens*: a simple, efficient and universal gene transfer technology that permits marker gene elimination. *Plant Cell Rep* 21:778–784
- Mliki A, Staub JE, Sun ZY, Ghorbel A (2001) Genetic diversity in melon (*Cucumis melo* L.): an evaluation of African germplasm. *Genet Res Crop Evol* 48:587–597
- Molina RV, Nuez F (1996) The inheritance of organogenic response in melon. *Plant Cell Tissue Organ Cult* 46:251–256

- Molina RV, Nuez F (1997) Sexual transmission of the in vitro regeneration capacity via caulogenesis of *Cucumis melo* L. in a medium with a high auxin/cytokinin ratio. *Sci Hort* 70:237–241
- Monforte AJ, Garcia-Mas J, Arus P (2003) Genetic variability in melon based on microsatellite variation. *Plant Breed* 122:153–157
- Monforte AJ, Oliver M, Gonzalo MJ, Alvarez JM, Dolcet-Sanjuan R, Arus P (2004) Identification of quantitative trait loci involved in fruit quality traits in melon (*Cucumis melo* L.). *Theor Appl Genet* 108:750–758
- Morales M, Roig E, Monforte AJ, Arús P, Garcia-Mas J (2004) Single-nucleotide polymorphisms detected in expressed sequence tags of melon (*Cucumis melo* L.). *Genome* 47:352–360
- Morales M, Orjeda G, Nieto C, van Leeuwen H, Charpentier M, Monfort A, Arús P, Puigdomenech P, Aranda MA, Dogimont C, Bendahmane A, Garcia-Mas J (2005) A physical map covering the *nsy* locus that confers resistance to Melon necrotic spot in melon (*Cucumis melo* L.). *Theor Appl Genet* 111:914–922
- Moreno V, Garcia-Sogo M, Granel I, Garcia-Sogo B, Roig LA (1985) Plant regeneration from calli of melon (*Cucumis melo* cv. ‘Amarillo Oro’). *Plant Cell Tissue Organ Cult* 5:139–146
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–493
- Muruganantham M, Ganapathi A, Selvaraj N, Anand RP, Vasudevan A, Vengadesan G (2002) Adenine sulphate and L-glutamine enhance multiple shoot induction from cotyledon explants of melon (*Cucumis melo* L. cv Swarna). *Cucurbit Genet Coop Rep* 25:22–24
- Nakata E, Staub J, López-Sesé A, Katzir N (2005) Genetic diversity of Japanese melon cultivars (*Cucumis melo* L.) as assessed by random amplified polymorphic DNA and simple sequence repeat markers. *Genet Res Crop Evol* 52:405–419
- Naudin CV (1859) Essai d’une monographie des espèces et des variétés du genre *Cucumis melo*. *Ann Sci Nat Bot* 11:5–87
- Nehring RB, Ecker JR (2004) Ethylene response in seedling growth and development In: Davies P (ed) *Plant hormones: biosynthesis, signal transduction, action!* Kluwer, Dordrecht, pp 350–368
- Neuhausen SL (1992) Evaluation of restriction fragment length polymorphism in *Cucumis melo*. *Theor Appl Genet* 83:379–384
- Nicaise V, German-Retana S, Sanjuan R, Dubrana MP, Mazier M, Maisonneuve B, Candresse T, Caranta C, LeGall O (2003) The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the potyvirus lettuce mosaic virus. *Plant Physiol* 132:1272–1282
- Niedz RP, Smith SS, Dunbar KB, Stephen CT, Murakishi HH (1989) Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo*. *Plant Cell Tissue Organ Cult* 18:313–319
- Nieto C, Morales M, Orjeda G, Clepet C, Montfort A, Truninger V, Sturbois B, Arus P, Caboche M, Puigdomenech P, Pitrat M, Dogimont C, Garcia-Mas J, Aranda M, Bendahmane A (2006) An *eIF4E* allele confers resistance against an uncapped and non-polyadenylated RNA carmovirus in melon. *Plant Journal* 48:452–462.
- Noguera FJ, Capel J, Alvarez JI, Lozano R (2005) Development and mapping of a codominant SCAR marker linked to the *andromonoecious* gene of melon. *Theor Appl Genet* 110:714–720
- Norton JD, Granberry DM (1980) Characteristics of progeny from interspecific cross of *Cucumis melo* L. with *C. metuliferus* E. Mey. *J Am Soc Hort Sci* 105:174–180
- Oliver JL, Garcia-Mas J, Cardús M, Pueyo N, López-Sesé AI, Arroyo M, Gómez-Paniagua H, Arús P, de Vicente CM (2001) Construction of a reference linkage map of melon. *Genome* 44:836–845
- Oridate T, Oosawa K (1986) Somatic embryogenesis and plant regeneration from suspension callus culture in melon (*Cucumis melo* L.). *Jpn J Breed* 36:424–428
- Oridate T, Atsumi H, Ito S, Araki H (1992) Genetic difference in somatic embryogenesis from seeds in melon (*Cucumis melo* L.). *Plant Cell Tissue Organ Cult* 29:27–30
- Palmer J (1982) Physical and gene mapping of chloroplast DNA from *Atriplex triangularis* and *Cucumis sativus*. *Nucleic Acids Res* 10:1593–1605

- Papadopoulou E, Little HA, Hammar SA, Grumet R (2005) Effect of modified endogenous ethylene production on sex expression, bisexual flower development and fruit production in melon (*Cucumis melo* L.). Sex Plant Reprod 18:131–142
- Park SO, Crosby KM, Huang RF, Mirkov TE (2004a) Identification and confirmation of RAPD and SCAR markers linked to the *ms-3* gene controlling male sterility in melon (*Cucumis melo* L.). J Am Soc Hort Sci 129:819–825
- Park Y, Katzir N, Brotman Y, King J, Bertrand F, Havey M (2004b) Comparative mapping of ZYMV resistances in cucumber (*Cucumis sativus* L.) and melon (*Cucumis melo* L.). Theor Appl Genet 109:707–712
- Pauquet J, Burget E, Hagen L, Chovelon V, Le Menn A, Valot N, Desloire S, Caboche M, Rousselle P, Pitrat M, Bendahmane A, Dogimont C (2004) Map-based cloning of the *Vat* gene from melon conferring resistance to both aphid colonization and aphid transmission of several viruses. Proc Cucurbitaceae 2004, 8th Eucarpia Meeting on Cucurbit Genetics and Breeding, Olomouc, pp 325–329
- Percheipied L, Dogimont C, Pitrat M (2005a) Strain specific and recessive QTLs involved in control of partial resistance to *Fusarium oxysporum* f. sp. *melonis* race 1.2 in a recombinant inbred line population of melon. Theor Appl Genet 111:65–74
- Percheipied L, Bardin M, Dogimont C, Pitrat M (2005b) Relationship between loci conferring downy mildew and powdery mildew resistance in melon assessed by QTL mapping. Phytopathology 95:556–565
- Périn C, Hagen LS, Giovinazzo N, Besombes D, Dogimont C, Pitrat M (2002a) Genetic control of fruit shape acts prior to anthesis in melon (*Cucumis melo* L.). Mol Gen Genomics 266:933–941
- Périn C, Hagen LS, de Conto V, Katzir N, Danin-Poleg Y, Portnoy V, Baudracco-Arnas S, Chadoeuf J, Dogimont C, Pitrat M (2002b) A reference map of *Cucumis melo* based on two recombinant inbred line populations. Theor Appl Genet 104:1017–1034
- Périn C, Gomez-Jimenez MC, Hagen L, Dogimont C, Pech JC, Latché A, Pitrat M, Lelièvre JM (2002c) Molecular and genetic characterization of a non-climacteric phenotype in melon reveals two loci conferring altered ethylene response in fruit. Plant Physiol 129:300–309
- Perl-Treves R, Galun E (1985) The *Cucumis* plastome: physical map, intragenic variation and phylogenetic relationships. Theor Appl Genet 71:417–429
- Perl-Treves R, Zamir D, Navot N, Galun E (1985) Phylogeny of *Cucumis* based on isozyme variability and its comparison with plastome phylogeny. Theor Appl Genet 71:430–436
- Pitrat M (1991) Linkage groups in *Cucumis melo* L. J Hered 82:406–411
- Pitrat M (2002) 2002 Gene list for melon. Cucurbit Gene Coop Rep 25:76–93
- Plages JN (1997) L'avenir des variétés génétiquement modifiées pour la résistance aux virus (un exemple développé par Limagrain). C R Acad Agric Fr 83:161–164
- Ramachandran C, Seshadri VS (1986) Cytological analysis of the genome of cucumber (*Cucumis sativus* L.) and muskmelon (*Cucumis melo* L.). Z Pflanzenzüch 86:25–38
- Rose JKC, Bennett AB (1999) Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. Trends Plant Sci 4:176–183
- Rose JKC, Saladié M, Catalá C (2004) The plot thickens: new perspectives of primary cell wall modification. Curr Opin Plant Biol 7:296–301
- Roustan JP, Latché A, Fallot J (1992) Enhancement of shoot regeneration from cotyledons of *Cucumis melo* by AgNO<sub>3</sub>, an inhibitor of ethylene action. J Plant Physiol 140:485–488
- Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). Plant J 32:1067–1075
- Sato-Nara K, Yuhashi K, Higashi K, Hosoya K, Kubota M, Ezura H (1999) Stage- and tissue-specific expression of ethylene receptor homolog genes during fruit development in muskmelon. Plant Physiol 120:321–329
- Sauton A (1988) Doubled haploid production in melon (*Cucumis melo* L.). In: Risser G, Pitrat M (eds) Proc Eucarpia Meeting 'Cucurbitaceae 88', Avignon, pp 119–128



- Sauton A, Dumas de Vaulx R (1987) Obtention de plantes haploïdes chez le melon (*Cucumis melo* L.) par gynogenèse induite par du pollen irradié. *Agronomie* 7:141–147
- Serrano R, Culianz-Macia FA, Moreno V (1999) Genetic engineering of salt and drought tolerance with yeast regulatory genes. *Sci Hort* 78:261–269
- Shiboleth YM, Arazi T, Wang Y, Gal-On A (2001) A new approach for weed control in a cucurbit field employing an attenuated potyvirus-vector for herbicide resistance. *J Biotechnol* 92:37–46
- Silberstein L, Kovalski I, Brotman Y, Perin C, Dogimont C, Pitrat M, Klingler J, Thompson G, Portnoy V, Katzir N, Perl-Treves R (2003) Linkage map of *Cucumis melo* including phenotypic traits and sequence-characterized genes. *Genome* 46:761–773
- Silva JA, da Costa TS, Luchetta L, Marini LJ, Zanuzo MR, Nora L, Nora FR, Twyman RM, Rom-baldi CV (2004) Characterization of ripening behaviour in transgenic melons expressing an antisense 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene from apple. *Postharvest Biol Technol* 32:263–268
- Staub JE, Box J, Meglic V, Horejsi TF, McCreight JD (1997) Comparison of isozyme and random amplified polymorphic DNA data for determining intraspecific variation in *Cucumis*. *Genet Res Crop Evol* 44:257–269
- Staub JE, Danin-Poleg Y, Fazio G, Horejsi T, Reis N, Katzir N (2000) Comparative analysis of cultivated melon groups (*Cucumis melo* L.) using random amplified polymorphic DNA and simple sequence repeat markers. *Euphytica* 115:225–241
- Staub JE, Dane F, Reitsma K, Fazio G, López-Sesé A (2002) The formation of test arrays and a core collection in cucumber using phenotypic and molecular marker data. *J Am Soc Hort Sci* 127:558–567
- Staub JE, López-Sesé AI, Fanourakis N (2004) Diversity among melon landraces (*Cucumis melo* L.) from Greece and their genetic relationships with other melon germplasm of diverse origins. *Euphytica* 136:151–166
- Stepansky A, Kovalski I, Perl-Treves R (1999) Intraspecific classification of melons (*Cucumis melo* L.) in view of their phenotypic and molecular variation. *Plant Syst Evol* 217:313–332
- Sujutha VS, Seshadri VS, Srivastava KN, More TA (1991) Isozyme variation in muskmelon (*Cucumis melo* L.). *Ind J Genet Plant Breed* 51:438–444
- Szabo Z, Gyulai G, Humphreys M, Horvath L, Bittsanszky A, Lagler R, Heszky L (2005) Genetic variation of melon (*C. melo*) compared to an extinct landrace from the Middle Ages (Hungary) I. rDNA, SSR and SNP analysis of 47 cultivars. *Euphytica* 146:87–94
- Tabei Y, Kanno A, Igarashi I, Nishio T (1987) Plant regeneration from cotyledon protoplasts of *Cucumis melo* L. *J Jpn Soc Hort Sci* 56:236–237
- Tabei Y, Kanno T, Nishio T (1991) Regulation of organogenesis and somatic embryogenesis by auxin in melon, *Cucumis melo* L. *Plant Cell Rep* 10:225–229
- Tabei Y, Nishio T, Kanno T (1992) Shoot regeneration from cotyledonary protoplast of melon (*Cucumis melo* L. cultivar charentais). *J Jpn Soc Hort Sci* 61:317–322
- Tabei Y, Oosawa K, Nishimura S, Watanabe S, Tsuchiya K, Yoshioka K, Fujisawa I, Nakajima K (1994) Environmental risk-evaluation of the transgenic melon with coat protein gene of cucumber mosaic-virus in a closed and semiclosed greenhouse. *Breed Sci* 44:207–211
- Taler D, Galperin M, Benjamin I, Cohen Y, Kenogsbuch D (2004) Plant *eR* genes that encode photorespiratory enzymes confer resistance against disease. *Plant Cell* 16:172–184
- Van Leeuwen H, Monfort A, Zhang HB, Puigdomenech P (2003) Identification and characterisation of a melon genomic region containing a resistance gene cluster from a constructed BAC library. Microcolinearity between *Cucumis melo* and *Arabidopsis thaliana*. *Plant Mol Biol* 51:703–718
- Vorster I, Jansen van Rensburg W (2004) The rediscovery of leafy vegetable boosts appreciation for diversity. International Plant Genetic Resources Institute (IPGRI), Rome, p 41
- Wang YH, Thomas CE, Dean RA (1997) A genetic map of melon (*Cucumis melo* L.) based on amplified fragment length polymorphism (AFLP) markers. *Theor Appl Genet* 95:791–797
- Ward BL, Anderson RS, Bendich AJ (1981) The mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). *Cell* 25:793–803
- Wilkinson JQ (1997) Biotech plants: from lab bench to supermarket shelf. *Food Technol* 51:37–42

- Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ (1997) A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. *Nat Biotechnol* 15:444–447
- Yadav RC, Saleh MT, Grumet R (1996) High frequency shoot regeneration from leaf explants of muskmelon. *Plant Cell Tissue Organ Cult* 45:207–214
- Yahyaoui FEL, Wongs-Aree C, Latché A, Hackett R, Grierson D, Pech JC (2002) Molecular and biochemical characteristics of a gene encoding an alcohol acyl transferase involved in the generation of aroma volatile esters during melon ripening. *Eur J Biochem* 269:2359–2366
- Yamagata H, Yonesu K, Hirata A, Aizono Y (2002) TGTCA motif is a novel *cis*-regulatory enhancer element involved in fruit-specific expression of the cucumisin gene. *J Biol Chem* 277:11582–11590
- Yamamoto M, Miki T, Ishiki Y, Fujinami K, Yanagisawa Y, Nkagawa H, Ogura N, Hirabayashi T, Sato T (1995) The synthesis of ethylene in melon fruit during the early stage of ripening. *Plant Cell Physiol* 36:591–596
- Yashiro K, Iwata H, Akashi Y, Tomita KO, Kuzuya M, Tsumura Y, Kato K (2005) Genetic relationship among East and South Asian melon (*Cucumis melo* L.) revealed by AFLP analysis. *Breed Sci* 55:197–206
- Yetisir H, Sari N (2003) A new method for haploid muskmelon (*Cucumis melo* L.) dihaploidization. *Sci Hort* 98:277–283
- Yoshioka K, Hanada K, Harada T, Minore Y, Oosawa K (1993) Virus resistance in transgenic melon plants that express the cucumber mosaic virus coat protein gene and in their progeny. *Jpn J Breed* 43:629–634



# I.10 Apple

A. M. IBANEZ and A. M. DANDEKAR<sup>1</sup>

## 1 Introduction

### 1.1 Apple Botany

Apple belongs to the genus *Malus* within the *Rosaceae* family, which includes some of the most commercially prominent fruit crops: pears, plums, peaches, nectarines, prunes, cherries, apricots, strawberry, raspberry, and blackberries. The genus *Malus* consists of at least 20–30 different species, including most of the domestic cultivars derived from interspecific hybridization due to self-incompatibility. This self-incompatibility promotes cross-pollination between compatible cultivars and makes apple fruit set dependent upon insect pollen vectors during flowering. Successful pollination of the pistil within the flower leads to formation of the best quality apples, which typically have 7–10 seeds. The generally accepted scientific name for apple is *Malus × domestica* Borkh. (Korvan and Skirvin 1984). It is also commonly designated as *Malus x domestica* or *Malus domestica* Borkh. (Phipps et al. 1990).

### 1.2 *Malus × domestica*

Apple cultivation dates back to a few centuries B.C., to the Greeks and Romans, who in turn spread apples through Asia and Europe. Its introduction to the Americas by European colonists began in the 16th and 17th centuries. Spanish priests grew apples at their missions in Chile and California. Spanish and Portuguese settlers introduced apples to settlements in suitable temperate zones of South America. European settlers brought apple seeds to establish orchards in eastern North America. Apples grow well from northern Ohio to eastern Canada. The first apple orchard in New England was recorded in the 1620s and 1630s, and apples soon became an important component in the New England farmstead. *Malus × domestica* is now cultivated widely in temperate latitudes and in high elevations in the tropics on all continents except Antarctica. For a review of the taxonomic classification and a brief history see Luby (2003).

---

<sup>1</sup> Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA, e-mail: amdandekar@ucdavis.edu

Many horticultural practices such as budding, grafting, hedging, and selection were developed in medieval times, when apples were grown around religious houses. The earliest known scientific breeding program for apple is that of Thomas Andrew Knight (1759–1835).

### 1.3 Breeding and Genetics

Apple has a haploid chromosome number of  $x = 17$ , but triploids, tetraploids, and hexaploids have been reported. Major breeding objectives are increased yield, quality, and range of harvest dates, and decreased chemical input. Breeding has also benefited from the discovery and evaluation of useful traits in other related plant species. Initially, the major emphasis was on developing resistance to diseases and pests, traits important to growers involved in fruit production. However, fruit quality and novelty have become important for varieties to sustain market share. Plant transformation can provide a way to introduce 'any gene from any source,' thereby substantially enlarging the range of germplasm available for crop improvement.

#### 1.3.1 Rootstocks

A rootstock constitutes the root system and a small proportion of the lower trunk of most apple trees, and is used to sustain scions of selected apple cultivars that have been grafted or budded onto them. Rootstocks have been used for more than 2000 years to invigorate apple scions. Rootstocks are produced either as seedlings or through vegetative propagation. Most apple rootstock clones are descended from *Malus* species, including *M. prunifolia* Wild., *M. baccata* (L.), Borkh., *M. pumila* (Malling series), *M. sylvestris* Miller, *M. micromalus*, *M. niedzwetskyana* ('Red Standard'), *M. floribunda*, *M. × robusta* ('Robusta 5') and *M. × domestica* ('Northern Spy') (Ferree and Carlson 1987; Webster 2002). The requirements for an ideal rootstock include long-term graft compatibility, freedom from viral and bacterial diseases caused by *Agrobacterium* or *Erwinia*, ease of propagation, reliable nursery performance, dwarfing ability to induce precocious and consistent cropping, resistance/tolerance to biotic and abiotic stresses, and freedom from suckers and burr knots (Webster and Wertheim 2003).

In 1917 the East Malling Research Station (UK) established the first rootstock breeding program, and in 1920 a series of rootstocks resistant to woolly apple aphid (WAA) (*Erisoma lanigerum* Hausmann) were developed. The first series was called 'Merton Immune', and this was followed by a 'Malling Merton' series, which is the best known and most propagated rootstock to date (Wertheim 1998; Webster and Wertheim 2003; Brown and Maloney 2005). The Cornell University (Geneva, USA) apple rootstocks breeding program, established in 1968, seeks to develop excellent rootstocks with resistance to biotic and abiotic stresses including fire blight caused by *Erwinia amylovora* (Bur-

rill). In 1998, it became a joint program of the United States Department of Agriculture–Agricultural Research Service (USDA–ARS) and Cornell University (Janik et al. 1996). The Cornell Geneva series is designated as ‘Geneva’ or the G series, and is well known for its excellent resistance to fire blight. Norelli et al. (2003) compared the resistance of Geneva rootstocks with other apple rootstocks when inoculated with the differentially virulent strain E4001a and other highly aggressive *E. amylovora* strains. They also evaluated the resistance of the Geneva rootstocks and advanced selections of the breeding program as rootstocks of grafted trees grown under orchard conditions. The more fire blight-resistant rootstocks were Geneva (G.) 11, G.16, G.30, G.66, Pillnitzer Au51-11, Malling 7, and several breeding selections. In 2003, G.11, G.16, G.30, and G.66 were released for commercial sale. In the USA, national rootstock testing trials (NC140) have been organized to evaluate rootstock–scion interactions as a function of location (Robinson et al. 2003). Rootstock breeding programs are also selecting for resistance to specific root diseases, e.g., black root rot (*Xylaria mali* Fromme), which is limited to southern USA, *Rosellina* root rot [*Rosellina necatrix* (Hart.) Berl.], which infects trees in India and California, and *Sclerotium rolfsii* Sacc., which is found in Israel (Janik et al. 1996).

The Canadian rootstock program in Quebec is breeding for hardiness, yield efficiency, precocity, dwarfing ability, and ease of propagation (Khanizadeh et al. 2000). In Japan, rootstock selection has focused on dwarfing character and resistance to crown rot and WAA (Wertheim 1998; Oraguzie et al. 2003). Recently Oraguzie et al. (2005), using molecular markers, fingerprinted a subset of rootstock clones maintained at the apple gene-bank of NIFTS (Morioka, Japan). Their objectives were to develop a DNA fingerprint for each rootstock clone, to verify and confirm parentage of hybrids, open-pollinated (op) clones, and sub-clones, and to determine the genetic diversity and relatedness of clones from different countries. Other countries with apple rootstock breeding programs are Germany and Sweden (Wertheim 1998), Poland (Jakubowski and Zagaja 2000), Romania, and the Czech Republic (Brown and Maloney 2005). For a complete review of apple rootstocks, see Wertheim (1998) and Webster and Wertheim (2003).

### 1.3.2 Scions

Apple scions are still a major focus of breeding efforts worldwide. One major breeding objective is to combine fruit quality with other horticulturally important traits such as disease resistance and plant architecture. Some common goals are resistance to apple scab (*Venturia inaequalis*) or powdery mildew (*Podosphaera leucotricha*), tree habit/architecture for high productivity, annual bearing (Fideghelli et al. 2003; Tartarini and Sansavini 2003), adaptation to extreme climates (Labuschagne et al. 2002, 2003; Sherman and Beckman 2003), and self-fertility (Broothaerts et al. 2004). Breeding for durable resis-

tance to pests and diseases has been stimulated by use of new tools such as molecular markers, mapping, and cloning. Promising progress has been made towards breeding for durable scab resistance (Liebhard et al. 2003; Gy-gax et al. 2004; Kellerhals et al. 2004) and WAA (Sandanayaka et al. 2003). It is possible to incorporate combinations of functionally different resistance genes into a single cultivar (pyramiding of resistance genes). Several genes are being tested to enhance host resistance to fire blight in apple (Norelli et al. 2003). In Japan, researchers are breeding for resistance to *Alternaria alternata* (Oraguzie et al. 2003). One new direction is to breed low-allergenic apple cultivars for consumers who have allergic reactions to apples and are unable to enjoy the health benefits of apple consumption (Laimer et al. 2005; Newcomb et al. 2006). Development of apples with unique qualities, appearance, and flavor is necessary to enhance marketing opportunities, e.g., apples with enhanced resistance to flesh browning after cutting are desirable for processing, and apples with red flesh, full surface russet, unusual shape, color, flavor, or enhanced edible quality are also sought after. Several excellent reviews provide a more comprehensive discussion of fruit traits and apple scion breeding objectives and accomplishments (Brown and Maloney 2005).

## 2 Economic Importance

On the basis of recent estimates, apple is the fourth most widely grown fruit in the world, with the others being citrus, bananas, and grapes (citrus, 105,077,728 metric tons; bananas, 72,588,562 metric tons; grapes, 65,733,393 metric tons; apple, 63,407,407 metric tons) ((FAOSTAT 2005). Apple production occurs over a total area of 5,205,026 ha and is worth more than \$23.5 billion (value based on 5-year average US prices for all sales; FAOSTAT 2005; US Apple Statistics 2005). The total production of 9,837,114,000 lb in the USA is worth about \$1.7 billion, of which the majority is the cultivars 'Red Delicious', 'Golden Delicious', 'Gala', 'Fuji', 'Granny Smith', 'McIntosh' and 'Rome Beauty' (US Apple Statistics 2005). Production in the USA over the past 10 years has been steady, with reasonably good consumer acceptance of individual apple cultivars, including a range of new cultivars. This trend would support the genetic transfer of particular traits, e.g., pest and disease resistance, or improved qualities such as fruit color or nutrition, to leading apple cultivars. The success of apple varieties such as 'Fuji' indicates that new apple cultivars with moderate appearance but superior quality can compete against apple cultivars with superior appearance or color, but variable quality.

### 3 Current Research and Development

#### 3.1 Tissue Culture

##### 3.1.1 Micropropagation

Micropropagation techniques have been developed for many fruit species to assist rapid vegetative propagation of elite clones (Jain and Isihii 2003). Apple rootstocks have been micropropagated in Canada, Italy, France, and the USA, but its cost is not commercially competitive with stoolbed production and other nursery-based vegetative propagation methods. Production of micropropagated plantlets is not always efficient and, in some instances, may not warrant large investments. However, adventitious shoot proliferation has demonstrated high potential for production in apple and other woody species (Pierik 1991; Famiani et al. 1994). Small plantlets require specific and expensive management until they are rooted, hardened, and ready for final delivery (Debergh and Read 1991; Kitto 1997). Sucurani et al. (2001) suggested synthetic seed (synseed) as a partial solution to this problem that combines the advantages of cloning (uniformity and sanitation of the propagated material) with those of true seed (ease of delivery, conservation, and manipulation). Encapsulated plant tissues are converted to plantlets with a high efficiency of up to 70%, even after a few months of cold storage (Capuano et al. 1998). Since micropropagated leaves and shoot clusters maintain their regenerative potential after fragmentation and encapsulation, apple micropropagation can be mechanized via an organogenesis pathway (Sucurani et al. 2001).

A significant expense for most culture media is the solidifying agent, an expensive tissue culture grade agar typically obtained from a family of red macro algae (*Rhodophyta*) that is known to inhibit growth and development of some cultures. A general problem related to agar type is hyperhydricity and necrosis of tissue. Attempts have been made to identify suitable alternative gelling agents for use during the rooting stage of apple. Druart (1997) reported that 90–100% of *Malus × domestica* Borkh. cv. Compact Spartan shoots root on vermiculite when treated with auxin (IBA). Mohan et al. (2004) grew microcuttings of the apple rootstock Marubakaido (*Malus prunifolia* Borkh.) in a rooting medium composed of half-strength Murashige and Skoog salts and vitamins, 3% (w/v) sucrose, 0.49  $\mu$ M indol-3-butyric acid, and sugarcane bagasse (a low cost alternative for traditionally used agar-gelled medium). The resulting microcuttings had 22% longer roots, were 20% higher, and had 63% more roots than agar-grown microcuttings. Blends of agar/galactomannan have also been tested for micropropagation of Marubakaido apple rootstocks (Lucyszyn et al. 2005). Results from in vitro experiments indicated enhanced proliferation of apple shoots. There were fewer hyperhydric shoots in modified gel-medium, with the additional advantage of reduced cost. Furthermore, micropropagation of apple rootstocks has been improved by use of a dou-

ble phase medium and the addition of arginine to stimulate shoot elongation (Litwinczuk 2002). An automated, low-cost bioreactor system has also been tested for micropropagation of apple rootstock M.9 EMLA. Nodal cuttings were cultured hydroponically for 30 days, resulting in more than 90% of the plants rooting and acclimatizing successfully. The plantlets maintained genetic uniformity, as determined from RADP patterns of greenhouse-grown control plants (Chakrabarty et al. 2003).

### 3.1.2 *Micrografting to Eliminate Viruses*

Viruses can cause considerable economic loss to apple growers. At present there is no effective therapy for infected plants in the field, and spread is controlled by phytosanitary agencies (Laimer 2003; Laimer et al. 2005). The most important viral pathogens of apple are Apple chlorotic leaf spot virus (ACLSV), Apple stem grooving virus (ASGV), Apple stem pitting virus (ASPV), and the isometric or bacilliform viruses Prunus necrotic ringspot virus (PNRSV), Apple mosaic virus (ApMV), Prune dwarf virus (PDV), and Tomato ring spot virus (TmRSV). In vitro grafting of apical meristems, or micrografting, was initially developed to obtain pathogen-free citrus (Murashige and Tucker 1969; Navarro et al. 1975). Since then, it has also been employed to obtain virus-free apple plants (Alskieff and Villermur 1978). A range of methods, including heat therapy, treatment with tetracycline, meristem-tip culture, and combinations of these procedures are used to produce virus-free plants for breeding or as part of national quarantine programs to ensure disease-free material (James et al. 1997; James 1999, 2001).

Serological and nucleic acid-based assays have been developed to screen horticultural crops for graft-transmissible agents, particularly viruses and phytoplasmas. Substantial progress in the development of these methods has been made over the past decade. Serology was traditionally used to identify specific viruses. This technique was replaced by enzyme-linked immunosorbent assay (ELISA), which allowed qualitative and quantitative analysis. ELISA has been used to identify infections with ACLSV, ASPV, ApMV, and ASGV (Karesová and Paprstein 2001). Double antibody sandwich (DAS)-ELISA has also been used, because of its greater sensitivity, broader reactivity, and convenience (Mullis et al. 1986; Saiki et al. 1988) for simultaneous detection of ACLSV and ASGV (Corvo and Barros 2001). Nucleic acid-based assays commenced with the development of PCR in the mid-1980s (Mullis et al. 1986; Saiki et al. 1988), and were rapidly adapted to identify pathogens through detection of their genetic material. Reverse transcriptase-PCR (RT-PCR) was developed to detect pathogens with an RNA genome, e.g., ASPV (Kundu 2001). Several variations of RT-PCR have been developed, including nested-, one-step-, multiplex-, and real-time-RT-PCR. Nested-PCR is designed for high specificity, while multiplex-PCR allows the concurrent identification of viruses in plants with mixed infections in a single PCR experiment. An alternative approach to

virion purification is immuno-capture (IC), referred to as IC-RT-PCR (Wetzel et al. 1991; Nolasco et al. 1993; Nemechinov et al. 1995; Rowhani et al. 1995), which was used for the detection of ASGV (James 2001; Kirby et al. 2001) and ACLSV (Corvo and Barros 2001; Yoshikawa et al. 2001). Menzel et al. (2003) developed multiplex RT-PCR-ELISA for simultaneous detection of ACLSV, ASPV, ApMV, and ASGV.

## 3.2 Somatic Cell Genetics

### 3.2.1 Somatic Embryogenesis

Somatic embryogenesis was induced in apple calli isolated from tissue explants of seedlings, flower buds, and petals, and grown on nutrient media with supplements. Embryos were diploid, triploid, tetraploid, polyploid, and aneuploid, but diploid ones predominated (Mehra and Sachdeva 1984). Five media with different concentrations and types of phytohormones were evaluated for optimum shoot quality of the apple rootstock 'M4'. Media containing BA and IBA produced the maximum number of shoots desirable for transplanting and acclimatization (David et al. 1985).

Since haploid apple plants are especially useful in breeding programs for production of homozygous material, two antimitotic agents, colchicine and oryzalin, were compared for efficiency in inducing chromosome doubling of in vitro grown haploid apple shoots. Oryzalin was preferable to colchicine for chromosome doubling in haploid apple shoots (Bouvier et al. 1994).

In spite of sustained efforts, it was not possible to obtain transformed apple plants via somatic embryogenesis (James et al. 1989, 1993; Yao et al. 1995), but it was possible with organogenesis (Belaizi et al. 1991; D'Angeli et al. 2001). Paul et al. (1994) used secondary somatic embryogenesis as an alternative process for high and rapid regeneration of 'Golden Delicious' apple, and Daigny et al. (1996) used the same process for 'Gloster 69'. Primary somatic embryos were produced from cotyledon-derived cultures of immature zygotic embryos. These somatic embryos were multiplied by secondary somatic embryogenesis (SSE) on medium with different plant growth regulator combinations. In addition, the effects of explant source, somatic embryo size, and type and concentration of carbohydrates and gelling agents on SSE were investigated (Ma et al. 1990; Daigny et al. 1996).

Significant progress has been made during the last 5 years in plant regeneration via organogenesis and somatic embryogenesis for economically important tree species. These advances will facilitate more rapid testing and release of trees improved through biotechnology (Narender et al. 2005).

Recently, Paek et al. (2005) proposed use of a bioreactor for automation of micropopagation via organogenesis or somatic embryogenesis of *Anoectochilus*, apple, *Chrysanthemum*, garlic, ginseng, grape, *Lilium*, *Phalaenopsis*, and potato, as a possible way of reducing production costs.



### 3.2.2 Cryopreservation

Cryopreservation of woody plant twigs at  $-160^{\circ}\text{C}$  was proposed by Sakai (1960). Methods have evolved such that cryopreservation has become the only reliable option for long-term preservation of woody species germplasm. Cryopreservation successfully avoids intracellular ice crystal formation, which causes irreversible damage to cell membranes, destroying their semipermeability in a wide range of tissues and organs such as cell suspensions, embryogenic callus, pollen, meristematic tissues, seeds, and embryo axes (Panis and Lambardi 2005).

Application of 'vitrification/one-step freezing' to woody species was facilitated by the PVS2 solution introduced for cryopreservation of *Citrus sinensis* nucellar cells (Sakai et al. 1990). PVS2 has been used for cryopreservation of shoot tips from several economically important hardwood species (e.g., *Malus*, *Pyrus*, *Prunus*, *Populus*, and *Vitis vinifera*). For *Malus* spp., in addition to 'vitrification/one-step freezing', the slow-cooling technique is still used, with a shoot tip survival rate of up to 92% (Zhao et al. 1999). Encapsulation/dehydration has been used for cryopreservation of shoot tips from nine different genera of hardwood species, among which are *Malus*, *Pyrus*, and *Prunus*. A shoot-tip survival of 80% or more has been reported for half of the species cryopreserved by an encapsulation/dehydration procedure (Lambardi and DeCarlo 2003). Recently, combining these procedures, a new technique named encapsulation/vitrification has successfully been applied to apple shoot tips (Paul et al. 2000; Sakai 2000). The procedure combines encapsulation of explants with application of a vitrification mixture.

Recently, a cryopreservation method for dormant vegetative buds based on Sakai's (1960) original procedure was applied by Towill et al. (2004) to 1915 accessions of apple. After retrieval from storage and grafting onto rootstocks, more than 90% of accessions showed a survival rate higher than 30%. One of the first woody plant germplasm repositories to use cryopreservation technology is the National Seed Storage Laboratory (NSSL) at Fort Collins (USA), which has about 2100–2200 accessions of dormant apple buds (Engelmann 2004; Panis and Lambardi 2005).

### 3.3 Transgenic Technology

Apple transformation was first reported by James et al. (1989), who used the *Agrobacterium*-mediated transformation of leaf disks from the apple cultivar 'Greensleeves' with the binary vector pBin6. A detailed procedure for 'Greensleeves' transformation was subsequently published, which established an excellent experimental system for apple (James and Dandekar 1991). Genes transferred via *Agrobacterium*-mediated transformation were stably incorporated and inherited in a simple Mendelian fashion (James et al. 1994, 1996). Transformation was possible because key tissue culture procedures such as

micropropagation and adventitious bud formation had been developed previously in apple. Adventitious bud formation on leaf disks is a vital component of any apple transformation system, because it maintains clonal identity of the cultivar being transformed. Unfortunately, regeneration is highly variable among different apple cultivars. Factors that may affect regeneration frequency include inorganic medium components, type and concentration of phytohormones, physiology of the explant source, and cultivar.

A significant problem of low transformation efficiency that was reported in early studies still applies to some apple cultivars (James et al. 1989; James and Dandekar 1991). Studies from the late 1980s and the 1990s pinpointed several factors that influence transformation efficiency, including the type and physiological characteristics of explant tissue, the strain of *Agrobacterium* sp., and the design of plasmid vectors (James et al. 1988; Dandekar et al. 1990; James and Dandekar 1991; De Bondt et al. 1994, 1996; Sriskandarajah et al. 1994; Sriskandarajah and Goodwin 1998). *Agrobacterium* strains containing pTiBo542 or pTiC58 and their derivatives were found to be most virulent on apple (Dandekar et al. 1990; Martin et al. 1990; De Bondt et al. 1994, 1996). *Agrobacterium* virulence could be enhanced either genetically, by introducing additional copies of the virulence genes (Dandekar et al. 1990), or physiologically, by growing the bacterium under conditions that induce the virulence genes (James et al. 1993). Addition of sugars such as glucose to the cocultivation medium also stimulated virulence (De Bondt et al. 1994). Transformation was highly variable among different apple cultivars, possibly because of differences in infectability or integration of T-DNA (De Bondt et al. 1994; Puite and Schaart 1996). The source and physiological status of the explant were important for transformation, i.e., leaves from rooted shoots (James et al. 1990), age of explant (De Bondt et al. 1994), type of leaf wounding (Norelli et al. 1996), explants from etiolated shoots (Liu et al. 1998), and conditioning of explants in liquid medium (Sriskandarajah and Goodwin 1998). Selection of transformed tissue is another critical stage: the optimum concentration of antibiotic varied with apple cultivar and type of gelling agent. The antibiotic kanamycin is the primary selective agent in most studies, and the gelling agents were agar, gelrite, or a combination of the two (James et al. 1989; Maheswaran et al. 1992; Norelli and Aldwinckle 1993; Yepes and Aldwinckle 1994). Different antibiotic combinations must be tested to optimize selection of transformants and remove persistent *Agrobacterium* sp. (Norelli and Aldwinckle 1993; Yepes and Aldwinckle 1994; Hammerschlag et al. 1997). *Agrobacterium* genes involved in plant transformation can now be more specifically defined and manipulated to improve transformation efficiency (Tzfira and Citovsky 2002; Gelvin 2003).

The first field trial of transgenic apple plants was conducted in California in the spring of 1992 (James and Dandekar 1991; Dandekar A.M. permit no. 91-218-03 of the US Department of Agriculture's Animal Plant Health Inspection Service; Dandekar et al. 2004). Subsequently, many more trials have been conducted in the USA, and a few in the UK and New Zealand. These trials have indicated no adverse effects of transformation, and normal fertile plants

were produced. Yield and quality trials have not yet been conducted. Public acceptance is a major limiting factor that has greatly dampened commercial enthusiasm for marketing a genetically modified organism (GMO) such as apple. Thus essential field trials that extensively test interesting genes in apple have not yet occurred.

### 3.3.1 Genetic Transformation

The 21st century poses challenges for apple growers and nurseries, as increased demand for tree fruit requires novel production practices that sustain yield and quality, while minimizing damage to the environment and the food chains vital to human health. Additionally, rising costs of land and labor provide an incentive to grow high value crops such as tree fruit. Sustaining supply and demand depends on development and deployment of new technologies, including biotechnology. The technology to produce transgenic plants can have an important, powerful impact on some immediate disease and pest problems of tree crops, and could reduce dependence on chemical pesticides and fungicides. However, commercial success in the long term will depend upon the impact of this technology on providing viable options for improving quality and nutrition. Unfortunately, negative public acceptance of GMO products has greatly reduced development of these technologies in recent years.

### 3.3.2 Objectives

Genetic transformation and rapid propagation of elite cultivars are useful in improving apple agronomic traits such as disease and pest resistance, productivity, and yield, along with quality traits such as shelf life, ripening, and nutrition. Plant transformation in apple is also useful to find solutions that target human health, environmental, and ecological concerns.

### 3.3.3 Protocol

Apple transformation is predominantly accomplished with an *Agrobacterium* vector; this is the most common and useful tool for introducing novel DNA sequences into most plant species. In apple, *Agrobacterium* has provided a reproducible system for transformation. This has been made possible by linking transformation with highly efficient, reproducible in vitro regeneration systems (James et al. 1988; Welander 1988; Fasolo et al. 1989; Predieri et al. 1989). Transformation experiments utilize disarmed *Agrobacterium* strains containing binary vectors such as pBIN6 (Bevan 1984) and pCGN (McBride and Summerfelt 1990). The vectors are incorporated into the appropriate *Agrobacterium* strain via electroporation (Wen-jun and Forde 1989). Leaf explants are the target for most apple cultivars (James and Dandekar 1991; Dandekar et al. 2006). Expanded leaves from rapidly proliferating shoot cultures are used for

the preparation of explants. These explants are infected with the appropriate *Agrobacterium* strain, cocultivated, and subjected to shoot regeneration in the presence of antibiotic selection. Shoots from regeneration media are tested for rooting in the presence of selection. The plants are then allowed to acclimate, transferred to the greenhouse, and then to the field. It takes from 9 months to 1.5 years to go from transformation of explants to a plant in the field. A detailed protocol for the apple transformation has been published (James and Dandekar 1991; Dandekar et al. 2006).

### 3.3.4 *Regeneration*

Regeneration has been reported in several different apple cultivars and rootstocks, including 'McIntosh,' 'Triple Red Delicious' (Fasolo et al. 1989), 'M26,' 'M25,' 'M9,' and 'Greensleeves' (James 1987; James et al. 1988). Transgenic apple plants have been regenerated in several cultivars and rootstocks, including 'Delicious' (Sriskandarajah et al. 1994; Puite and Schaart 1996; Maximova et al. 1998; Sriskandarajah and Goodwin 1998), 'Elstar' (Puite and Schaart 1996), 'Gala' (Yao et al. 1995; Puite and Schaart 1996; Maximova et al. 1998), 'Greensleeves' (James et al. 1989, 1996; Maximova et al. 1998), 'M26' (Lambert and Tepfer 1992; Maheswaran et al. 1992; Norelli et al. 1994; Holefors et al. 1998), 'McIntosh' (Bolar et al. 1997), and 'Pink Lady' (Sriskandarajah and Goodwin 1998).

## 3.4 Molecular Genetics

### 3.4.1 *Gene Cloning*

Gene cloning in apple has intensified in the last two decades until now, with more than 250,000 EST sequences in GenBank, the cloning of any gene of interest is feasible. The focus has shifted from obtaining genes from their protein sequence to obtaining genes by virtue of the similarity of their DNA sequence. One way to evaluate the molecular genetic data is to view it in a trait-specific manner. Several groups have focused on factors affecting fruit ripening, which is central to apple fruit quality. This is a process in which ethylene, a plant growth regulator, plays an important role. Ethylene is also involved in other plant processes, including senescence and response to biotic and abiotic stress (Ecker and Davis 1987; McKeon and Yang 1987; Reid 1987; Kieber and Ecker 1993; Ecker 1995). Ethylene mediates expression of specific genes involved in ripening (Theologis 1994; Fluhr and Mattoo 1996) and stress (Kieber and Ecker 1993; Ecker 1995; Jackson 1997). Ethylene biosynthesis is mediated by two enzymes, 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), which have attracted the attention of several research groups. The ACS enzyme has been purified from apple (Yip et al. 1991) and the cDNA was also isolated

(Dong et al. 1991). Layyee and Knighton (1995) reported the sequence of the full-length apple ACS cDNA (Md-ACS1-1, *M. domestica* ACS). Sunako et al. (1999) identified an additional allele of the ripening gene encoding ACS (Md-ACS1-2) in apple fruit that is associated with long shelf life. ACO has also been isolated and characterized (Dong et al. 1992; Ross et al. 1992). Castiglione et al. (1999) suggested that at least two allelic forms of ACC oxidase exist. Tomato cDNA of ACS (Sato and Theologis 1989; Van Der Straeten et al. 1990; Huang et al. 1991) and ACO (Hamilton et al. 1991; Spanu et al. 1991) was cloned and found to be similar to the apple ethylene pathway, as both tomato and apple are climacteric fruits. In tomato, cDNA from ACS/ACO expressed in the antisense orientation caused decreased ethylene biosynthesis and delayed ripening (Hamilton et al. 1990; Oeller et al. 1991; Theologis 1994). Transgenic apple plants in which ACS and ACO genes have each been silenced revealed that many flavor and texture traits are under ethylene control (Dong et al. 1991, 1992; Dandekar et al. 2004; Defilippi et al. 2004, 2005a,b). Currently, transgenic apple plants with altered ethylene biosynthesis are being field-tested (Dandekar et al. 2004). Ethylene biosynthesis is detailed in Sect. 3.5.2.

Sorbitol, the sugar alcohol of glucose, is a major photosynthetic product in apple and other members of the family *Rosaceae*, such as *Malus*, *Pyrus*, *Prunus*, and *Sorbus* (Bielecki 1982; Loescher 1987). Sorbitol is also the primary translocatable carbohydrate and storage carbohydrate in these plants. Sorbitol is synthesized in mature leaves and translocated to growing tissues such as fruits and young leaves, where it is converted to fructose (Webb and Burley 1962; Bielecki 1969; Zimmermann and Ziegler 1975). Since apple is one of the most significant fleshy fruit species, there is interest in understanding sorbitol's role in growth and productivity of apple plants, and how it is synthesized and degraded. The cDNA coding for sorbitol-6-phosphate (S6PDH) has been cloned in apple (Kanayama et al. 1992, 1996; Kanayama and Yamaki 1993). Expression of sense cDNA encoding S6PDH in transgenic tobacco plants resulted in sorbitol accumulation, suggesting that S6PDH is the rate-limiting step in sorbitol synthesis in apple (Tao et al. 1995). Sorbitol is then translocated to sink tissues via the phloem. The transport mechanism of sugar alcohols such as sorbitol has been identified and these alcohols participate in the unloading step in fruit tissues (Noiraud et al. 2001; Gao et al. 2003; Zhang et al. 2004). To define and clarify the role of sorbitol accumulation, transgenic plants were created that were suppressed or upregulated for sorbitol synthesis through alteration of the leaf S6PDH concentration (Teo et al. 2006). Recently, Kanamaru et al. (2004) showed that increased sucrose accompanied the decreased sorbitol in transgenic apple leaves (*Malus domestica* Borkh. cv. 'Orin') suppressed for S6PDH. An indepth study by Cheng et al. (2005) provided evidence that the compensatory relationship between sorbitol and sucrose in transgenic apple plants downregulated for S6PDH does not affect the photosynthetic assimilation rate. For more information on sorbitol and its biosynthesis see Sect. 3.5.1.

Polyphenol oxidase (PPO; EC 1.10.3.1) is a copper-containing enzyme that catalyzes enzymatic browning of fruit, producing brown pigments and reducing quality of fruits and vegetables. Enzymatic browning also causes loss of polyphenols, important antioxidants in our diet. Murata et al. (2001) obtained transgenic apples carrying an antisense PPO gene. One transgenic apple callus line had half the amount and activity of PPO of untransformed callus. Antisense methods should be useful for regulating expression of PPO and studying its function, to the benefit of the food industry and consumers. Since PPO is an important enzyme in disease and pest resistance, antisense expression should be limited to fruit tissue. More details on PPO are given in Sect. 3.5.4.

Apple allergens have gained prominence because of health concerns and because allergens are inducible by stress, pathogens, and fruit ripening (Laimer et al. 2005). Atkinson et al. (1996) isolated the cDNA clone pAP15, which encodes a homologue of a class of stress- and pathogenesis-related cDNAs, and found that it is highly homologous to a class of allergenic proteins from apple. Northern analysis indicated that pAP15 is related to ripening, with an accumulation of homologous mRNA coincident with ethylene production during ripening. Vanek-Krebitz et al. (1995) cloned and sequenced Mal d 1, the major allergen from apple, and related it immunologically with Bet v I, the major birch pollen allergen. Studies comparing the concentrations of apple allergens in different tissues revealed that most of the apple allergen (Mal d 3) is in the fruit peel (Fernandez-Rivas and Cuevas 1999). In 2000, a major allergen protein was isolated and classified as a pathogen-related protein (Pühringer et al. 2000). Ypr10, a promoter of allergen Mal d 1, was inducible by stress and pathogens (Hoffmann-Sommergruber 2002). Pühringer et al. (2003) reported that the protein MdAp, encoded by a single gene, was a putative binding partner for the apple Mal d 1 protein. This protein is classified as a pathogen-related (PR-10) protein, but its biological function is still unknown. Mal d 2 apple allergen, a thaumatin-like protein which belongs to protein family PR5, was isolated and characterized as an antifungal protein against *Fusarium oxysporum* and *Penicillium expansum* (Hsieh et al. 1995; Krebitz et al. 2003). In 2002, after cloning a cDNA gene encoding for Mal d 3, the protein was classified as a non-specific lipid-transfer protein (LTP) (Diaz-Perales et al. 2002). LTPs are classified as pathogenesis-related (PR) protein PR-14 (Salcedo et al. 1999; Van Loon and Van Strien 1999), which, unlike other apple allergens, is heat resistant (Salcedo et al. 2004). LTPs are also associated with plant adaptation to abiotic stress, such as cold and salt (Kader 1996; Douliez et al. 2000; Salcedo et al. 2004). The SAFE project, a multidisciplinary European Consortium whose objective is to develop strategies for reducing fruit allergies, characterized, cloned, and sequenced four apple allergens (Mal d 1 to Mal d 4). Apple allergen variants were also identified (Hoffmann-Sommergruber 2005; see also Sect. 3.5.5).

Although several major scab-resistance genes have been known for some time (Williams and Kuc 1969), only one, the Vf gene, has been incorporated



into commercially available cultivars. Belfanti et al. (2004) reported cloning the resistance gene *HcrVf2*, derived from a wild *Malus* species, to transform the scab-susceptible apple cultivar 'Gala'. Four independent transformed lines were resistant to apple scab (*Venturia inaequalis*). *HcrVf2* is one of at least three resistance genes mapping at the *Vf* locus. The influence of the *HcrVf1* and *HcrVf4* genes on scab resistance must be investigated to determine whether *HcrVf2* is the only gene that triggers a resistance response. The cloning of an apple scab resistance gene will allow further investigation of the resistance mechanism, and represents a step toward gene therapy (restoring resistance where lost) of scab-susceptible cultivars that currently dominate areas of apple production (Baldi et al. 2004; Belfanti et al. 2004).

Self-incompatibility is a mechanism that prevents self-fertilization in flowering plants (de Nettancourt 1977). Self-incompatibility in *Solanaceae*, *Rosaceae* and *Scrophulariaceae* is gametophytically controlled by a single polymorphic locus, termed the S-locus, which produces a pistil-specific glycoprotein with ribonuclease activity (S-RNase). In this type of self-incompatibility, pollen-tube growth is inhibited within the style when an S-allele carried by pollen matches one of the two S-alleles carried by the pistil. There is a strong interest in self-fertility in many fruits, because self-pollination ensures more consistently high production yields than cross-pollination. However, true commercial self-fertile apple cultivars do not exist (Broothaerts et al. 2004). To date, 10 S-RNAs have been identified and cloned as *S*<sub>2</sub>-, *S*<sub>3</sub>-, *S*<sub>4</sub>-, *S*<sub>5</sub>-, *S*<sub>7</sub>-, *S*<sub>9</sub>-, *S*<sub>24</sub>-, *S*<sub>26</sub>-, *S*<sub>27</sub>-, and *S*<sub>25</sub>-RNA (Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996; Verdoodt et al. 1998; Kitahara et al. 1999, 2000; Matsumoto and Kitahara 2000; Van Nerum et al. 2001; Kitahara and Matsumoto 2002). The *S*<sub>25</sub>-RNase gene in the pistil, responsible for the self-incompatibility response within the apple *Malus x domestica* Borkh., was cloned from the 'McIntosh' cultivar (Kitahara and Matsumoto 2002). Broothaerts et al. (2004) produced a self-fertile apple resulting from S-RNase gene silencing. Transgenic plants and controls were grown over a period of 3 years in the greenhouse to control self- and cross-pollination of flowers. Transgenic lines produced normal fruits and seeds after selfing. In contrast, controls produced much less fruit following self-pollination (Broothaerts et al. 2004).

The juvenile period in some apples is long; some apple cultivars can flower in their fourth year, while others require 10 years to flower. The first transgenic 'Greensleeves' produced flowers in its fifth year (James et al. 1996). Reduced juvenility facilitates evaluation and characterization of transgenes in breeding programs. Transgenic 'Royal Gala' apple (*Malus x domestica*) was transformed with a mutant *Arabidopsis* acetolactate synthase (*asl*) gene conferring resistance to the herbicide chlorsulfuron, a *uidA* gene coding for  $\beta$ -glucuronidase (GUS), and a neomycin phosphotransferase II (*nptII*) gene conferring kanamycin resistance. One-year-old transgenic apple trees were grafted onto the dwarfing rootstock 'Malling 9'. Rapid flowering was achieved by growing transgenic trees under controlled greenhouse conditions; 85% produced flowers and fruit within the next year (Yao et al. 1999).



### 3.4.2 Marker Assisted Selection

Molecular markers and other DNA-based technologies (cDNA and genomic sequencing, microarrays and other expression analyses, gene mapping and associated genomics tools) will be important in future breeding and genetic engineering programs to speed discovery and manipulation of genes that affect commercial traits such as disease resistance, pest resistance, abiotic stress factors, grafting compatibility, growth architecture, nutritional properties, and processing and storage qualities. The molecular determination of internal quality traits of apple (e.g., flavor, texture, and color) and fruit safety (e.g., allergens) can also be addressed by biotechnological tools. These same tools will also help plant breeders select new varieties more rapidly.

Molecular markers are needed for more efficient breeding of specific traits; for example, molecular markers associated with apple scab (*Venturia inaequalis*) resistance can assist in selection of apple cultivars with several functionally different resistances, and reduce the risk of resistance breakdown (Gygax et al. 2004; Kellerhals et al. 2004). Pyramiding resistance genes in an apple cultivar is not an easy task. First, resistance genes must be available, preferably in advanced selections, and then molecular markers linked to those genes must be developed. DNA markers linked to the genes of interest are required to select seedlings with the desired combination of resistance alleles (Gianfranceschi et al. 1996). Tartarini et al. (1999) identified six scab resistance genes (*Vf*, *Vr*, *Vb*, *Va*, *Vm* and *Vbj*) from crab apples. For all of these genes, molecular markers have been developed: *Vf* (Koller et al. 1994; Vinatzer et al. 2004), *Vr* (Hemmat et al. 2002), *Vb* and *Va* (Hemmat et al. 2003), *Vm* (Cheng et al. 1998) and *Vbj* (Gygax et al. 2004). Two other apple scab resistance genes, *Vx* and *Vr2*, with associated molecular markers, have been identified (Hemmat et al. 2002; Patocchi et al. 2004). Among these genes, however, only *Vf* has been studied intensively.

Oraguzie et al. (2005) applied molecular markers to identify valuable genetic resources. Using seven simple sequence repeat (SSR) markers, they established the genetic identity and relationships of 66 rootstock clones representing a subsample of an apple rootstock collection from different countries maintained at the NIFTS genebank in Morioka, Japan. Their objectives were to (1) develop a DNA fingerprint for each rootstock clone, (2) verify/confirm parental lines of hybrids, open-pollinated (op) clones, and subclones, and (3) determine the genetic diversity and relatedness of clones from different countries. Their results suggest that SSR markers may not differentiate bud sport mutants or subclones from their original parents, but are very reliable for parental identification/verification of controlled crosses, chance seedlings, and cultivars/clones. However, more SSR markers are needed for a finer resolution of genetic relationships and parental identification.

Newcomb et al. (2006) developed an EST (expressed sequence tag) database from various tissues of apple, focusing on fruit tissues of *Malus x domestica* cv. 'Royal Gala'. This kind of database, combined with techniques such as

microarrays, can be used to select candidate genes that are implicated in particular crop traits. Furthermore, ESTs have been identified as useful sources of both SSRs (also known as microsatellites) (Morgante et al. 2002) and SNPs (single-nucleotide polymorphisms) (Rafalski 2002), both useful markers for creating genetic maps in plants.

### 3.5 Functional Genomics

#### 3.5.1 Carbohydrate Metabolism in Apple

Apple plants accumulate sorbitol in addition to sucrose and starch. Sorbitol, the sugar alcohol of glucose, is the predominant sugar found in apple. It is synthesized in mature leaves and translocated to fruit, where it is converted to fructose. Sorbitol is widely distributed in nature and can be found in species of bacteria, insects, animals, yeasts, algae, fungi, and higher plants (Touster and Shaw 1962; Bielecki 1982). However, certain woody members of the family *Rosaceae*, including *Malus*, *Pyrus*, *Prunus*, and *Sorbus* spp., are unique in the plant kingdom in their ability to synthesize, accumulate, and degrade sorbitol (Bielecki 1982; Loescher 1987). In apple, sorbitol is the major photosynthetic product translocated from mature leaves to growing tissues such as fruits and young leaves (Webb and Burley 1962; Bielecki 1969; Zimmermann and Ziegler 1975).

At the biochemical level, sorbitol metabolism occurs is controlled by two significant rate-limiting reactions. One forms sorbitol-6-phosphate from glucose-6-phosphate via aldose-6-phosphate reductase (EC 1.1.1.200, referred to here as sorbitol-6-phosphate dehydrogenase, S6PDH) in photosynthetic tissues (mature leaves). The second converts sorbitol to fructose via sorbitol dehydrogenase (SDH) in sink tissues (fruit and young developing leaves). Evidence that these two enzymes are critical steps in sorbitol metabolism is as follows:

1. The labeling pattern of photosynthate in mature apricot leaves suggests that sorbitol synthesis occurs through the intermediate sorbitol-6-phosphate (Bielecki and Redgwell 1977; Ridgwell and Bielecki 1978; Negm and Loescher 1981).
2. S6PDH has been purified from leaves of apple (Kanayama and Yamaki 1993, 1994) and loquat (Hirai 1981), and enzyme activity has been detected in leaves of pear, peach, apricot (Negm and Loescher 1981), and eight other plant species from the three subfamilies of *Rosaceae* (Hirai 1981), showing that this enzyme is widespread in plants that synthesize sorbitol.
3. Unlike mature leaves which produce mainly [ $^{14}\text{C}$ ] sorbitol from  $^{14}\text{CO}_2$ , very young apricot leaves produce mainly [ $^{14}\text{C}$ ] sucrose and no [ $^{14}\text{C}$ ] sorbitol. Young apricot leaves, however, import sorbitol by translocation from older leaves (Bielecki and Redgwell 1985).
4. Seasonal changes in sorbitol concentration coincide with the amount of S6PDH (Hirai 1983; Yamaki and Ishikawa 1986; Sakanishi et al. 1998).

5. S6PDH from apple was purified and cDNA coding for this enzyme was cloned (Kanayama et al. 1992; Kanayama and Yamaki 1993). Expression of this cDNA in tobacco was sufficient for sorbitol synthesis (Tao et al. 1995). Expression of sense and antisense cDNA encoding S6PDH in apple results in suppression of sorbitol accumulation in transgenic apple plants, indicating that S6PDH is the key enzyme involved in sorbitol biosynthesis (Cheng et al. 2005; Kanamaru et al. 2004; Teo et al. 2006). The alteration in carbon budget due to silencing of sorbitol in leaves alters key apple fruit quality traits, including sugar-acid accumulation and starch distribution (Teo et al. 2006).
6. SDH was detected in apple fruit (Negm and Loescher 1979; Yamaki 1980), purified, and the cDNA was identified (Kanayama and Yamaki 1994, 1996; Yamada et al. 1998). SDH is highly regulated during apple fruit development (Yamaguchi et al. 1994, 1996; Nosarszewski et al. 2004).
7. Very young apple leaves contain SDH but no S6PDH activity, but mature leaves have mainly S6PDH (Negm and Loescher 1981). This difference was related to leaf carbohydrate levels and net photosynthesis, indicating that sorbitol metabolism in apple is tightly controlled and may relate to mechanisms that regulate partitioning of source and sink activity (Loescher et al. 1982; Gao et al. 2003).
8. A phosphatase enzyme (sorbitol-6-phosphatase) removes the phosphate moiety from sorbitol-6-phosphate to form translocated sorbitol (Grant and Ap Rees 1981).
9. In 1998 the genomic sequence of S6PDH from *Malus × domestica* cv. 'Greensleeves' (Bains et al. 1998) contained features that are highly conserved among various members of the family *Rosaceae*.

Taken together, these data provide evidence that in many members of the *Rosaceae*, sorbitol synthesis as a result of photosynthesis occurs exclusively through S6PDH, while utilization is primarily through SDH. Anabolic and catabolic pathways for sorbitol accumulation are different, and under separate genetic and metabolic control.

Partitioning of assimilated carbon limits both the rate and pattern of vegetative and reproductive growth in plants (Cheng et al. 2005; Teo et al. 2006). Carbon partitioning in apple was studied using transgenic 'Greensleeves' plants expressing antisense/sense S6PDH; these apples show alterations in sugar composition, starch distribution, and sugar-acid balance in fruit (Teo et al. 2006). These experiments could help genetically link key carbon metabolism pathways with the regulatory enzymatic steps. In turn, this information will clarify the relationship in apple between source organs that partition assimilated carbons such as sucrose and sorbitol, and various sink organs using these translocated sugars. Additionally, understanding these pathways and developing the means to manipulate them will not only impact the yield of biomass, but also allow manipulation of important quantitative apple traits.

Sorbitol may affect the ability of apple plants to respond to environmental stress. Several physiological roles are proposed for sugar alcohols in higher plants: they may act as compatible solutes, protectants to stabilize membranes, and carbon storage compounds (Schobert 1977; Lewis 1984). The most important role reported is that sugar alcohols serve as osmolytes in response to water, salt, and other abiotic stress (Wang and Stutte 1992; Tarczynski et al. 1993; Wang et al. 1995). Accumulation of sugar alcohols helps the cell gain more water through osmotic adjustment (Morgan 1984; Turner and Jones 1980). Much attention has been given to mannitol and its involvement in salt tolerance, but relatively little information exists on the importance of sorbitol in this area. It is implicated in osmotic adjustment to overcome decreasing water potential created by environmental stress in cherry (Ranney et al. 1991), drought stress in apple leaves (Wang and Stutte 1992; Wang et al. 1995, 1996), cold hardiness (Raese et al. 1978; Whetter and Taper 1966), increased photosynthesis via elevated CO<sub>2</sub> (Pan and Quebedeaux 1995), and protein stabilization during desiccation (Wimmer et al. 1997). Moing et al. (1997) suggested that sorbitol variability is related to the geographical origin of *Prunus* species. Transgenic apple plants expressing antisense transgenes that inhibit synthesis or degradation of sorbitol will provide genetic approaches to study some of these issues. Newcomb et al. (2006) presented an extensive set of ESTs, representing half of the expressed genes from apple. The data set contains SSR and SNP markers that will be useful for breeding, and many genes that can be tested directly for their roles in various crop traits. This gene set also forms the basis of an apple microarray that is being used to further identify genes encoding biosynthetic enzymes and their regulators.

### 3.5.2 Ethylene Biosynthesis in Apple

Ethylene is an important plant growth regulator that affects diverse plant processes including fruit ripening, senescence, and response to biotic and abiotic stress (Ecker and Davis 1987; McKeon and Yang 1987; Reid 1987; Kieber and Ecker 1993; Ecker 1995). At a molecular level, ethylene mediates expression of specific genes involved in ripening (Theologis 1994; Fluhr and Mattoo 1996) and stress (Kieber and Ecker 1993; Ecker 1995; Jackson 1997).

Physiological and biochemical characteristics of apple ripening are comprehensively reviewed by Hulme (1968, 1971). Much of the classical work on fruit ripening was done with apple. Kidd and West (1922) observed the characteristic rise in respiration when apples are detached from the tree and stored at normal ripening temperatures. They called the phenomenon 'climacteric' (Kidd and West 1925). In 1932, Kidd and West (1933) showed that vapors produced by ripe apples caused unripe apples to undergo the characteristic climacteric rise in respiration and thus ripen. Gane (1934, 1935) demonstrated that the active component of the vapors was ethylene. The gaseous hormone ethylene plays an important role in apple ripening, and there is an excellent correla-

tion between ethylene concentration in apple fruit and its shelf life and eating quality. Selections with inherently low ethylene production can be generated in progeny of a cross with a low ethylene producer as one parent (Stow et al. 1993). 1-amino cyclopropane-1-carboxylate (ACC) is a key intermediate in ethylene biosynthesis, and the rate-limiting step (Adams and Yang 1979; McKeon and Yang 1987; Dandekar et al. 2004). The biosynthetic pathway from methionine involves the enzyme ACC synthase, which converts S-adenosylmethionine (SAM) to ACC. ACC is then converted to ethylene by the ethylene-forming enzyme ACC oxidase.

cDNA of both ACC synthase (Sato and Theologis 1989; Van Der Straeten et al. 1990; Huang et al. 1991) and ACC oxidase (Hamilton et al. 1991; Spanu et al. 1991) has been cloned. The cDNA from these genes expressed in the antisense orientation causes decreased ethylene biosynthesis and delayed ripening (Hamilton et al. 1990; Oeller et al. 1991; Theologis 1994; Dandekar et al. 2004). The ACC synthase enzyme has been purified from apple (Yip et al. 1991) as well as its cDNA (Dong et al. 1991). ACC oxidase has also been characterized from apple (Fernandez-Maculet and Yang 1992) and its cDNA isolated (Dong et al. 1992). Alternative approaches to control ethylene concentration are through expression of a bacterial enzyme called ACC deaminase that metabolizes ACC (Klee et al. 1991), or through tissue-specific expression of SAM hydrolase (Good et al. 1994) or SAM decarboxylase (Mehta et al. 1997, 1999), which metabolizes SAM. The latter approaches render the precursors SAM or ACC unavailable for ethylene biosynthesis (Theologis 1994; Fluhr and Mattoo 1996).

Many physiological, genetic, and developmental factors influence the storage and eating quality of apples. Two indices are used to determine eating quality: visual appeal and physiological maturity, which includes acidity, ethylene or CO<sub>2</sub> concentration, soluble solids, flesh firmness, and starch content. These have been standardized for each cultivar and are used to predict harvest dates and determine storage time. Ethylene concentration is a key fruit quality trait that profoundly influences all maturity indices. Therefore, control of ethylene biosynthesis in apple also controls shelf life and eating quality. This is being investigated through expression of antisense mRNA and through expression of other genes and chemicals that interfere with ethylene synthesis. Transgenic apple plants with altered ethylene biosynthesis now in field tests include some expressing antisense/sense ACC synthase and others expressing antisense/sense ACC oxidase (Dandekar et al. 2004). The impact of suppressing ethylene action or biosynthesis on flavor metabolites, related enzymes, and precursor availability in apple peel and flesh tissue has been investigated (DeFilippi et al. 2004, 2005a, b). However, it will be some time before the impact of these approaches on apple shelf life and other quality traits can be fully determined.

### 3.5.3 Anthocyanin and Flavonol Biosynthesis

Skin color is an important trait in apples, with a deep red preferred commercially and aesthetically. Apple skin color is a blend of many components, including anthocyanins/flavanols, carotenoids, and chlorophyll. The yellow/green color of some cultivars is due to synthesis of chlorophyll and carotenoids in plastids. However, red apple color is due to synthesis of anthocyanins and flavanols that accumulate in the vacuole. Anthocyanin synthesis is highly regulated by the environment (Saure 1990). The exact chemical composition of apple skin pigments is known (Lancaster and Dougall 1992). Engineering skin color may be possible, as the basic pathways for pigment biosynthesis have been elucidated in flowers (Meyer et al. 1987; Mol et al. 1989). However, specific aspects of pigment synthesis must be determined in apple, for example, pathways for synthesis of cyanidin glycosides, proanthocyanidins like catechin, and flavanols such as quercetin glycoside. Some of this information is available (Lister et al. 1997). Additionally, skin color is highly regulated during fruit development and ripening (Lister et al. 1996; Kim et al. 2003). Therefore, modifications to apple skin color must be expressed in a pattern specific to the correct tissue and developmental stage. This process will require identification of specific regulatory sequences.

### 3.5.4 Polyphenol Constituents

Studies in 1997 showed that polyphenol constituents of fruits such as apple are more effective antioxidants *in vitro* than vitamins C and E, and thus may be more valuable for protection *in vivo*. Therefore, the active ingredient that makes “an apple a day keep the doctor away” may be the phytochemical components of the apple fruit, such as flavonoids, phenylpropanoids, and phenolic acids. All of these make important contributions to antioxidant activity in the human diet (Rice-Evans et al. 1997). Apple flavonols such as quercetin and flavones such as rutin may be important (Rice-Evans et al. 1997). As more is learned about the synthetic pathways for these compounds, their amounts can be modulated. Some of these compounds are also color pigments, so modification of their concentrations may also influence fruit color.

A significant problem with ‘fresh cut’ or ‘juice’ processed apples is enzymatic browning, which is easily observed in bruised and wounded fruit, and which causes widespread losses to the food industry. The browning reaction is an enzyme-catalyzed reaction, unlike Maillard non-enzymatic browning. It is catalyzed by polyphenol oxidase (PPO; EC 1.10.3.1), also known as catechol oxidase, phenolase, and *o*-diphenol oxygen oxidoreductase. This enzyme is widely distributed among terrestrial and aquatic plant species (Sherman et al. 1991). Another enzyme, tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), catalyzes both the orthohydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Lerch 1981).



This copper-containing enzyme is also widely distributed, and is responsible for synthesis of melanin pigments (Bell and Wheeler 1986; Walker and Ferrar 1998). While melanins have several important functions and properties (Bell and Wheeler 1986), little is known about PPO function and expression in plants. Recent evidence suggests that these enzymes may facilitate disease (Boss et al. 1995; Walker and Ferrar 1998) and pest (Steffens et al. 1994) resistance. PPO is highly regulated both developmentally and temporally in plants (Hunt et al. 1993; Kim et al. 2001), and different forms are present in the skin and flesh of apple fruit (S.P. Robinson, personal communication). Broothaerts et al. (2000) developed a rapid screening assay to detect PPO in transgenic plants of apple and tobacco.

Murata et al. (2001) transformed apple with the antisense PPO gene and obtained one transgenic apple callus line, in which the amount and activity of PPO were reduced to half that of non-transgenic callus. This antisense expression of PPO in apple is a potential approach to reduce enzymatic browning of apple fruit. Antisense expression must be targeted to fruit tissue to prevent interference with PPO expression elsewhere in the plant, where it may be important for disease/pest resistance.

### 3.5.5 *Allergens*

Apple, carrot, and potato allergens became of interest to medical researchers some two decades ago, when they observed that patients allergic to birch pollen (e.g., Bet v 1 and Bet v 2 birch pollen profilin), who were mainly from North America and central and northern Europe, frequently also had intolerance to some fruits and vegetables (Eriksson et al. 1982; Dreborg and Foucard 1983; Lowenstein and Eriksson 1983; Fritsch et al. 1997). Ebner et al. (1995) identified allergens in apple, pear, carrot, celery, and potato. Apple allergens cross-react immunologically with birch pollen allergens, and are potentially life-threatening for patients allergic to birch pollen. The symptoms are a local reaction of the upper aero-digestive tract mucosa (itching, inflammation, angioedema). Many patients also experience systemic symptoms (urticaria, asthma, anaphylactic shock) (Ebner et al. 1995; Hsieh et al. 1995). Apple allergens do not increase during storage and ripening, and are associated with stress and pathogen resistance (Hsieh et al. 1995). Fernandez-Rivas and Cuevas (1999) compared allergenicity of apple peel and pulp, and found that peel was more allergenic. Adverse reactions appeared more frequently and were more severe when the whole fruit was eaten. The primary apple allergens are identified as: (1) Mal d 1, a Bet v 1 homologue inducible by stress and pathogens, (2) Mal d 2, a compact, thaumatin-like protein with antifungal activity and high resistance to proteolysis, a characteristic that links this allergen to induction of severe food allergies, and (3) Mal d 3, a non-specific, heat stable, lipid-transfer protein also related to severe allergy symptoms; it accumulates in peel and is linked to abiotic stress and pathogen resistance



(Diaz-Perales et al. 2002; Hoffmann-Sommergruber 2005). Localization and distribution of major allergens in apple fruit was investigated. Mal d 1 and Mal d 2 were equally distributed throughout the pulp in all cultivars, while Mal d 3 was absent in pulp but clearly detectable in the peel (Marzban et al. 2005).

Up to 90% of patients with birch pollen allergy develop intolerance to fruits and/or vegetables, with at least two million US citizens affected (Fernandez-Rivas 2003). Apple is one of the most consumed fruits in the world, so apple allergens have been of interest to several research groups. One plant food allergy program used apple allergens as a model system to develop field-to-table strategies for reducing allergy incidence. A multidisciplinary consortium from seven European countries (Austria, Finland, Italy, the Netherlands, the UK, Spain, and Switzerland) is developing strategies to reduce the incidence of fruit allergies (Hoffmann-Sommergruber 2005). Four apple allergens (Mal d 1–4) were characterized and variants identified, cloned, and sequenced. Mal d 1 and 4 were clearly linked to birch pollen sensitization, but there was no clear link between Mal d 2 and d 3 and pollen sensitization. Changes in allergen characteristics during harvest, storage, and processing were investigated, as was the impact of agronomic practices. Allergen genes were mapped on a molecular linkage map. The biological function of Mal d 1 was studied using RNA interference. Consumer attitudes in northern, central, and southern Europe were also investigated.

### 3.5.6 *Other Quality Traits*

Texture is an important quality feature of eating apples, with a firm breaking texture being desirable. Understanding softening could allow better control of ‘mealiness’. In many climacteric fruits, dramatic changes in texture and cell wall structure take place during ripening, triggered by the autocatalytic synthesis of ethylene. Regulation of endopolygalacturonase in apple may also be involved in softening (Wu et al. 1993).

Controlling metabolic activity during storage is especially important to minimize abiotic problems such as watercore, bitter pit, and storage scald. Watercore-affected tissues are high in sorbitol, and the presumed mechanism is that sorbitol accumulation leads to cellular damage by osmotic lysis of cells. Sorbitol accumulation may be caused by sorbitol transport problems in tissues (Gao et al. 2005). Stress-induced metabolic activity in stored fruit can lead to formation of reactive oxygen species (ROS), resulting in problems such as storage scald. Oxidative stress is implicated in cellular damage in tissues under stressful conditions such as high light intensity, temperature extremes, salinity, heavy metals, herbicides, and toxins. An important ROS scavenging systems in plants is the enzyme superoxide dismutase (SOD). Transgenic plants with elevated SOD expression were protected from the damaging effects of ROS (Allen et al. 1997). SODs are a class of metalloproteins that catalyze dismutation

of the superoxide radical to hydrogen peroxide and oxygen; various isoenzyme forms are present in different fruit, including apple (Manganaris and Alston 1997).

## 4 Practical Applications of Transgenic Plants

### 4.1 Alternatives to Chemical Pesticides: Genes Encoding Insecticidal Proteins

Insects are a significant problem in leading apple production areas such as the Pacific Northwest in the USA. Codling moth (CM) (*Cydia pomonella*) is the major pest there and worldwide in apple-producing areas. It attacks fruit at the 1- to 2-cm stage, hence the name *codling* ('young one/baby'). Codling moth is a lepidopteran insect that lays eggs on fruit or leaf clusters near the fruit. Larvae feed on fruit and cause considerable economic damage. CM larvae do not feed on the surface but burrow into the apple fruit, which makes them difficult to control with chemical pesticides. Alternatives are needed that kill pests but avoid chemical pesticides. One such approach in tree crops is to use insect pest resistance genes from several sources (Escobar and Dandekar 2000).

The predominant strategy to engineer CM resistance in apple has been through expression of *Bacillus thuringiensis* (Bt) genes that encode insecticidal proteins. The insecticidal activity of *B. thuringiensis* resides in the bacterium as a parasporal crystalline inclusion body with one or more insecticidal crystal proteins (ICPs) (previously referred to as *delta-endotoxins*) (reviewed in Whiteley and Schnepf 1986; Schnepf et al. 1998; Höfte and Whiteley 1989). The insecticidal properties of crystal/spore suspensions of *B. thuringiensis* have been exploited commercially for almost 40 years in products such as Dipel (Abbot Laboratories), Javelin (Sandoz), Thuricide (Sandoz), and others. When the ICP is ingested by a target insect, it dissolves in the alkaline pH of the insect midgut and is acted upon by midgut protease(s), releasing the active N-terminal fragment of the insecticidal crystal protein (ICPF) and killing the insect (Höfte and Whiteley 1989). In vitro studies have shown that the ICPF binds with high specificity and affinity to specific cell receptors on the brush border membrane of midgut epithelial cells (Hofmann et al. 1988a, b; Van Rie et al. 1990; Schnepf et al. 1998). This binding correlates with formation of pores and membrane lesions that lead to swelling, leakage, and lysis of the epithelium, ultimately causing death of the insect through starvation and septicemia (Knowles and Ellar 1987; Schnepf et al. 1998). Non-target organisms do not possess the specific cell receptors, and as a result are unaffected by this protein.

ICP genes from different *Bacillus* species are categorized on according to the host range of their activity and DNA sequence homology (Höfte and Whiteley 1989; Crickmore et al. 1998). *cryIAC* and *cryIAB* are toxic to CM larvae (Vail et al. 1991). Initial studies on *cryIAC* expression in apple used unmodified gene

sequences obtained from *B. thuringiensis*. This approach was based upon the success achieved in tobacco (Vaeck et al. 1987) and tomato (Fischhoff et al. 1987) against tobacco hornworm and tobacco budworm. However, transgenic apple plants with wild-type *B. thuringiensis* cryIAC sequences had insufficient ICP to cause significant mortality of CM larvae (Dandekar et al. 1992). Similar results were obtained in walnut against CM larvae (Dandekar et al. 1994). A strategy to circumvent these problems by altering the gene through chemical synthesis was reported by Perlak et al. (1991). The gene product has an identical amino acid sequence; the major differences are at the nucleic acid level. This altered gene functioned very well in plants, with expression > 500-fold higher than the wild-type coding region (Perlak et al. 1991). Chemically synthesized versions of cryIAC have been introduced into apple, where they confer high levels of mortality to CM larvae (Dandekar et al., unpublished data). These trees are currently being field tested (USDA/APHIS permit no. 97-028-02r). Similar experiments have also been done in walnut (Dandekar et al. 1998) and persimmon (Tao et al. 1997), where chemically synthesized versions of cryIAC provided excellent protection against target insect larvae. Transgenic tissues expressing cryIAC protein at as low as 0.02% of total cellular protein produced a 100% mortality in CM larvae (Dandekar et al. 1998).

Genes encoding *B. thuringiensis* ICPs can be used against some other commercially significant lepidopteran pests of apple orchards. These include orange tortrix (*Argyrotaenia citrana*), apple pandemis (*Pandemis prysuana*), obliquebanded leafroller (*Chroistoneura rosaceana*), fruittree leafroller (*Archips argyrospila*), omnivorous leafroller (*Platynota sultana*), western tussock moth (*Orgyia vetusta*), fruit worms (*Orthosia hibisi*, *Amphipyra pyramidoides*), and leafminers (*Phyllonorycter* spp.). Typically, these lepidopteran insects are controlled by chemical pesticides and become problematic in organic orchards. However, *B. thuringiensis* ICPs display a strong differential toxicity, and it is important to check susceptibility of other caterpillars besides CM. All apple orchard locations are different, and may have a range of pests. Aphid, mite and scale outbreaks are usually associated with chemical pesticide use, but pesticides can also kill the predators of these insects. A great benefit of transgenic orchards expressing ICPs would be the greater opportunity to integrate biocontrol. Predators of mites, aphids, and scale would flourish in such an orchard because no generalized chemical pesticides would be sprayed. This would reduce both aphid, mite, and scale outbreaks and pesticide use.

## 4.2 Resistance Development and Management Strategies

Because of their persistence and stability in the ecosystem, trees expressing a single resistance gene may select for resistance in insect populations (Raffa 1989). Biopesticides containing *B. thuringiensis* ICPs enjoyed a 40-year or more record of commercial use with no evidence of resistance development. However, since the early 1990s there have been several findings of resistance

(Tabashnik et al. 1990, 1991; Ferre et al. 1991; Sims and Stone 1991). This has happened under post-harvest conditions with extreme selection pressure in stored grain products (McGaughey 1985; McGaughey and Johnson 1987; McGaughey and Beeman 1988), and under deliberate selection conditions in the laboratory (Stone et al. 1989; Sims and Stone 1991). Studies on the mechanism of resistance indicate that it involves a decrease in the binding affinity between the insect's midgut brush border membrane and the insecticidal protein (Van Rie et al. 1990; Ferre et al. 1991; MacIntosh et al. 1991). In most cases, resistance was recessive, suggesting that strategies involving refugia may prevent resistance development. In these strategies, untransformed plants are planted within the transgenic population to help maintain a viable susceptible population that breeds out resistance (Gould 1998; Roush 1998). Another resistance management strategy is to have plants express ICP at very high levels that kill not only susceptible insects, but also heterozygous resistant insects. The Environmental Protection Agency (EPA) in 1998 defined the high dose as 25 times the concentration required to kill 99% of susceptible insects (Gould 1998). In 1999, an incompletely dominant resistance allele was reported in *Ostrinia nubilalis* (European corn borer) (Huang et al. 1999). This is the first report of a dominant ICP resistance allele, and this type of resistance will be difficult to manage with the two strategies described. Dominant resistance to ICPs could be controlled by gene pyramiding: expressing additional insecticidal genes that do not share the same mode of action, or. Clearly, research is needed to clarify resistance mechanisms and then develop and deploy a management strategy for codling moth in apple.

### 4.3 Engineering Disease Resistance in Apple

Among the most significant apple diseases are scab, caused by the fungus *Venturia inaequalis*, and fire blight, caused by the bacterium. Both diseases spread in cool, moist conditions and cause significant losses worldwide. The two general strategies to obtain resistance to pathogens are (1) to clone disease resistance genes from resistant varieties and (2) to test individual genes that directly affect growth and multiplication of the pathogen. Resistance genes trigger a plant defense response through a gene-for-gene interaction (reviewed in Staskawicz et al. 1995; Maleck and Lawton 1998).

Resistance to apple scab, referred to as the *Vf* gene, is present in *Malus floribunda* and has been introgressed into several apple varieties, such as 'Prima'. In 1998, the map position of the *Vf* gene in a mapping population that included progeny from a cross between the apple cultivars 'Prima' and 'Fiesta' was determined (Maliapaard et al. 1998). Direct genetic approaches using simple sequence repeats have also been used to identify markers that link the *Vf* gene to unique DNA fragments (Gianfranceschi et al. 1998; Tartarini et al. 1999). About 16 markers have been identified from a molecular genetic analysis of 19 *Malus* × *domestica* (Borkh.) cultivars or selections with germplasm

from *M. floribunda* (Gianfranceschi et al. 1996, 1998). Tightly linked random amplified polymorphic DNA (RAPD) markers have also been developed for a *Vf* gene that gives resistance to five races of *Venturia inaequalis* (Hemmat et al. 1998). Once the *Vf* gene is cloned, it can be introduced into apple cultivars of choice to verify resistance to *V. inaequalis*. It is well known that the *Vf* scab resistance widely used in apple breeding programs can be overcome by specific races and strains of the fungus, especially in northern Europe (Kellerhals et al. 2004). Molecular markers are available to detect the *V<sub>m</sub>*, *V<sub>r</sub>*, and *V<sub>bj</sub>* scab resistance genes (Hemmat et al. 2002; Gyga et al. 2004). The *VstI* gene from *Vitis vinifera* L. is also being used to transform apple [*Malus × domestica* Borkh. cvs. 'Elstar' and 'Holsteiner Cox' (Szankowski et al. 2003)]. Alternative approaches to engineer resistance include expression of an antimicrobial peptide (Cubber et al. 2000), chitinase (Mehlenbacher 1995; Hanke et al. 2000), exochitinases (Bolar et al. 1998, 2001; Norelli et al. 1999, 2000), and endochitinases (Bolar et al. 1997, 1999, 2000, 2001). Transgenic apple plants expressing high levels of endochitinase are resistant to *V. inaequalis*, but are also stunted (Bolar et al. 1997). One promising strategy for breeding durable scab resistance is to combine several functionally different resistances in a cultivar (pyramiding of resistance genes) (Liebhard et al. 2003; Gyga et al. 2004; Kellerhals et al. 2004). Molecular markers and genetic linkage maps are needed to allow detection and analysis of major resistance genes and quantitative trait loci (QTL) contributing to the resistance of a genotype. Frey et al. (2004) developed a low-cost DNA extraction and a multiplex fluorescent PCR method for marker-assisted selection (MAS). The method can analyze up to eight markers in a single multiplex reaction. Systems have been established that allow switching from SCAR (sequence-characterized amplified region)-based screening to microsatellite-based screening on an automated fragment analyzer.

Resistance to fire blight, caused by *Erwinia amylovora* in apple, has been obtained via expression of genes encoding lytic peptides. Expression of the lytic peptide attacin E in the apple rootstock 'Malling 26' provided good resistance to the pathogenic bacterium (Norelli et al. 1994, 1996, 1999, 2000; Aldwinckle et al. 1999; Ko et al. 1999, 2002; Hanke et al. 2000). In addition to scion cultivars, rootstocks are particularly sensitive to *E. amylovora*. Unfortunately, the transgenic "Malling 26" turned out to be 'Malling 7', which is naturally resistant to fire blight. No major resistance genes have been found (Forsline and Aldwinckle 2002). Other genes used to induce fire blight resistance are  $\phi$  Ea1-depolymerase (Hanke et al. 2002), SB-37 (Aldwinckle et al. 1999; Norelli et al. 1999), T4 lysozyme (Aldwinckle et al. 1999; Hanke et al. 1999; Norelli et al. 1999; Ko et al. 2002), *hrpN* (harpin) (Kader et al. 1999), cecropin MB 39 gene (modified SB-37) (Liu et al. 1999, 2001), and *Shiva-1* (Norelli et al. 1999). There are also opportunities for breeding fire blight-resistant apple and pear cultivars by exploiting genetic variation in germplasm and by developing QTL markers (Kellerhals et al. 2004).

## 5 Conclusions and Future Challenges

Apple is a dominant fruit crop with a long history of genetic manipulation. Breeding and selection programs have provided commercially acceptable cultivars. However, manipulation of fruit quality is an important challenge. Apple fruit is an excellent model for tree fruit crops, as there is considerable useful information available on its physiology and biochemistry. With the advent of genomics and large-scale sequencing of cDNA, there is useful information on apple genes in GenBank, which provides a resource for study and manipulation of apple. Gene regulation of apple fruit development provides a system for study of fruit ripening physiology, and the resulting insights will help develop new tools to optimize fruit quality in the field and after harvest. Many genes can serve as markers to more rapidly breed new varieties of apples with novel quality and nutritional phenotypes. The new genetic tools can be used to study apple germplasm to better define the allelic variation of genes involved in fruit quality and resistance to disease and pests. Exploiting genetic resistance to disease and pests is important to create a safe growing and production environment that uses less chemicals and fossil fuel.

### 5.1 Emerging Opportunities

Apple is one of the most technologically advanced tree fruit crop species. Genetic engineering provides an unrealized opportunity for improvement of fruit quality and shelf life through manipulation of ethylene and sorbitol metabolism. The technology can reduce fruit allergens, making the crop safe for more consumers. Resistance to insect pests such as codling moth represents another unrealized opportunity to decrease dependence on harmful chemical pesticides. Expression of insecticidal proteins from Bt was never commercially realized, largely due to the public perception of the GMO issue. Plant codon-optimized Bt insecticides could eliminate many insect pest problems in apple, and allow better integration of insect management strategies to control others. Resistance to apple scab through breeding or transformation could eliminate the use of fungicides. Finally, genomics and, more importantly, functional genomics can provide a more fundamental understanding of apple biology, which will enable discovery of new genes to improve apple quality and productivity for the future.

## References

- Adams DO, Yang SF (1979) Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proc Natl Acad Sci USA* 76:170–174



- Aldwinckle HS, Norelli JL, Bolar JP, Ko K, Harman GE, Brown SK, Ko KS, Altman A, Ziv M, Izhar S (1999) Genetic engineering of disease resistance in apple fruit cultivars and rootstocks. *Curr Plant Sci Biotechnol Agric* 36:449–451
- Allen RD, Webb RP, Schake SA (1997) Use of transgenic plants to study antioxidant defences. *Free Rad Biol Med* 23:473–479
- Alskieff J, Villermur P (1978) Greffage in vitro d'apex sur des plantules decapitees de pommier (*Malus pumila* Mill.). *C R Acad Sci Serie D* 287:1115–1118
- Atkinson RG, Perry J, Matsuri T, Ross G, Macrae E (1996) A stress-, pathogenesis-, and allergen related cDNA in apple fruit is also ripening-related. *N Z J Crop Hortic Sci* 24:103–107
- Bains HS, Tao R, Uratsu SL, Dandekar AM (1998) Genomic nucleotide sequence of a NADP sorbitol-6-phosphate dehydrogenase gene from apple (accession no. AF057134) (PGR 98-193). *Plant Physiol* 118:1533
- Baldi P, Patocchi A, Zini E, Toller C, Velasco R, Komjanc M (2004) Cloning and linkage mapping of resistance gene homologues in apple. *Theor Appl Genet* 109:231–239
- Belaizi M, Paul H, Sangwan RS, Sangwan-Norreel BS (1991) Direct organogenesis from internodal segments of in vitro grown shoots of apple cv. Golden Delicious. *Plant Cell Rep* 9:471–474
- Belfanti E, Silberberg-Dilworth E, Tartarini S, Patocchi A, Barbieri M, Zhu J, Vinatzer BA, Gianfranceschi L, Gessler C, Sansavini S (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proc Natl Acad Sci USA* 101:886–890 ([www.pnas.org/cgi/doi/10.1073.pnas.0304808101](http://www.pnas.org/cgi/doi/10.1073.pnas.0304808101))
- Bell AA, Wheeler MH (1986) Biosynthesis and functions of fungal melanins. *Annu Rev Phytopathol* 24:411–451
- Bevan MW (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12:8711–8721
- Bielecki RL (1969) Accumulation and translocation of sorbitol in apple phloem. *Aust J Biol Sci* 22:611–620
- Bielecki RL (1982) Sugar alcohols. In: Loewus F, Tanner W (eds) *Encyclopedia of plant physiology*. New series, vol 13A. Springer, Berlin Heidelberg New York, pp 158–192
- Bielecki RL, Redgwell RJ (1977) Synthesis of sorbitol in apricot leaves. *Aust J Plant Physiol* 4:1–10
- Bielecki RL, Redgwell RJ (1985) Sorbitol versus sucrose as photosynthesis and translocation products in developing apricot leaves. *Aust J Plant Physiol* 12:657–668
- Bolar JP, Aldwinckle HS, Harman GE, Norelli JL, Brown SK (1997) Endochitinase-transgenic McIntosh apple lines have increased resistance to scab. *Phytopathology* 87:S10
- Bolar JP, Norelli JL, Aldwinckle HS, Harman GE, Brown SK (1998) Expression of an exochitinase gene from *Trichoderma harzianum* in transgenic apple lines. *Phytopathology* 88:S8–S9
- Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS, Altman A, Ziv M, Izhar S (1999) Expression of fungal chitinolytic enzymes in transgenic apples confers high levels of resistance to scab. *Curr Plant Sci Biotechnol Agric* 36:465–468
- Bolar JP, Norelli JL, Wong KW, Hays CK, Harman GE, Aldwinckle HS (2000) Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigour. *Phytopathology* 90:72–77
- Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic plants. *Transgenic Res* 10(6):533–543
- Boss PK, Gardner RC, Janssen BJ, Boss GS (1995) An apple polyphenol oxidase cDNA is up-regulated in wounded tissue. *Plant Mol Biol* 27:429–433
- Bouvier I, Fillon FR, Lepinase Y (1994) Oryzalin as an efficient agent for chromosome doubling of haploid apple shoots in vitro. *Plant Breed* 113:343–346
- Broothaerts W, Janssens GA, Proost P, Broekaert WF (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. *Plant Mol Biol* 27:499–511
- Broothaerts W, McPherson J, Li BC, Randall E, Lane WD, Wiersma PA (2000) Fast apple (*Malus × domestica*) and tobacco (*Nicotiana tabacum*) leaf polyphenol oxidase activity assay for screening transgenic plants. *J Agric Food Chem* 48:5924–5928



- Broothaerts W, Keulemans J, Van Nerum I (2004) Self-fertile apple resulting from S-RNase gene silencing. *Plant Cell Rep* 22:497–501
- Brown SK, Maloney KE (2005) *Malus × domestica* apple. In: Litz RE (ed) *Biotechnology of fruit and nut crops*. CABI International, Wallingford, pp 475–511
- Capuano G, Piccioni E, Standardi A (1998) Effect of different treatments on the conversion of M.26 apple rootstock synthetic seeds obtained from encapsulated apical and axillary micropropagated buds. *J Hort Sci Biotechnol* 73:299–305
- Castiglione S, Pirola B, Sala F, Ventura M, Pancaldi M, Sansavini S (1999) Molecular studies of ACC synthase and ACC oxidase genes in apple. *Acta Hort* 484:305–309
- Chakrabarty D, Hahn EJ, Yoon YJ, Paek PY (2003) Micropropagation of apple rootstock M.9 EMLA using bioreactor. *J Hort Sci Biotechnol* 78:605–609
- Cheng FS, Weeden NF, Brown SK, Aldwinckle HS, Gardiner SE, Bus VG (1998) Development of a DNA marker for *Vm*, a gene conferring resistance to apple scab. *Genome* 41:208–214
- Cheng L, Zhou R, Reidel EJ, Shaarkey TD, Dandekar AM (2005) Antisense inhibition of sorbitol synthesis without altering CO<sub>2</sub> assimilation in apple leaves. *Planta* 220:767–776
- Corvo LM, Barros MTF (2001) The use of a simplified DAS-ELISA procedure for large scale detection of *Apple chlorotic leaf spot* and *Apple stem grooving* viruses in apple trees over extended sampling periods. *Acta Hort* 550:269–274
- Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean C (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Molec Biol Rev* 62:807–813
- Cubber K, Broothaerts W, Lenaerts T, Keulemans J, Webster AD (2000) Progress in genetic transformation as a tool for increased disease resistance in apple. *Acta Hort* 525:309–316
- D'Angeli S, Lauri P, Caboni E, Dewitte W, Van Onckelen H (2001) Factors affecting in vitro shoot formation from vegetative shoot apices of apple and relationship between organogenic response and cytokinin localization. *Plant Biosyst* 135:95–100
- Daigny G, Paul H, Sangwan RS, Sangwan-Norreel BS (1996) Factors influencing secondary somatic embryogenesis in *Malus × domestica* Borkh. (cv. 'Gloster 69'). *Plant Cell Rep* 16:153–157
- Dandekar AM, Uratsu SL, Matsuta N (1990) *Agrobacterium*-mediated transformation of apple: factors influencing virulence. *Acta Hort* 280:483–494
- Dandekar AM, McGranahan GH, Uratsu SL, Leslie C, Vail PV, Tebbets SJ, Hoffman D, Driver J, Viss P, James DJ (1992) Engineering for apple and walnut resistance to codling moth. In: Brighton GBR (ed) *Proc Brighton Crop Protection Conf on Pests and Diseases*, Cambridge, pp 741–747
- Dandekar AM, McGranahan GH, Vail PV, Uratsu SL, Leslie CA, Tebbets JS, Hoffman DJ (1994) Low levels of expression of *cryIA(c)* sequences of *Bacillus thuringiensis* in transgenic walnut. *Plant Sci* 96:151–162
- Dandekar AM, McGranahan GH, Vail PV, Uratsu SL, Leslie CA, Tebbets JS (1998) High level of expression of full length *cryIA(c)* gene from *Bacillus thuringiensis* in transgenic somatic walnut embryos. *Plant Sci* 131:181–193
- Dandekar AM, Teo G, Defilippi BG, Uratsu SL, Passey AJ, Kader AA, Stow JR, Colgan RJ, James DJ (2004) Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. *Transgenic Res* 13:373–384
- Dandekar AM, Teo G, Uratsu SL, Tricoli D (2006) Apple (*Malus × domestica*). In: Wang L (ed) *Agrobacterium* protocols. *Methods in molecular biology*, vol 44. Humana Press, Totowa, New Jersey
- David ID, Turner KE, Lazaroff WR (1985) Propagation in vitro of the apple rootstock M4: effect of phytohormones on shoot quality. *Plant Cell Tissue Organ Cult* 4:55–60
- Debergh PC, Read PE (1991) Micropropagation. In: Debergh PC, Zimmerman RH (eds) *Micropropagation: technology and application*. Kluwer, Dordrecht, pp 1–14
- De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert WF (1994) *Agrobacterium*-mediated transformation of apple (*Malus × domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Rep* 13:587–593

- De Bondt A, Eggermont K, Penninckx I, Goderis I, Broekaert WF (1996) *Agrobacterium*-mediated transformation of apple (*Malus × domestica* Borkh.): an assessment of factors affecting regeneration of transgenic plants. *Plant Cell Rep* 15:549–554
- Defilippi BG, Dandekar AM, Kader AA (2004) Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus domestica* Borkh.) fruits. *J Agric Food Chem* 52:5694–5701
- Defilippi BG, Kader AA, Dandekar AM (2005a) Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Sci* 168:1199–1210
- Defilippi BG, Dandekar AM, Kader AA (2005b) Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues. *J Agric Food Chem* 53:3133–3141
- De Nettancourt D (1977) Incompatibility in angiosperms. In: Frankel R, Gal GAE, Linskens HF (eds) *Monographs on theoretical and applied genetics*. Springer, Berlin Heidelberg New York, pp 28–57
- Diaz-Perales A, Garcia Selles FJ, Barber D, Salcedo G (2002) cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. *Clin Exp Allergy* 32:87–92
- Dong J-G, Kim WT, Yip WK, Thompson GA, Li L, Bennett AB, Yang S-F (1991) Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. *Planta* 185:38–45
- Dong J-G, Olsen DB, Silverstone A, Yang S-F (1992) Sequence of a cDNA encoding for a 1-aminocyclopropane-1-carboxylate oxidase homolog from apple fruit. *Plant Physiol* 98:1530–1531
- Douliez JP, Michon T, Elmorjani K, Marion D (2000) Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J Cereal Sci* 32:1–20
- Dreborg S, Foucard T (1983) Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy* 38:162–172
- Druart P (1997) Optimization of culture media for in vitro rooting of *Malus × domestica* Borkh. cv. Compact Spartan. *Biol Plant* 39:67–77
- Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 95:962–969
- Ecker JR (1995) The ethylene signal transduction pathway in plants. *Science* 268:667–675
- Ecker J, Davis RW (1987) Plant defense genes are regulated by ethylene. *Proc Natl Acad Sci USA* 84:5202–5206
- Engelmann F (2004) Plant cryopreservation: progress and prospects. *In Vitro Cell Dev Biol-Plant* 40:427–433
- Eriksson NE, Formgren H, Svenonius E (1982) Food hypersensitivity in patients with pollen allergy. *Allergy* 37:437–443
- Escobar M, Dandekar AM (2000) Development of insect resistance in fruit and nut tree crops. In: Jain SM, Minocha SC (eds) *Molecular biology of woody plants*, vol 2. Kluwer, Dordrecht, pp 395–417
- Famiani F, Ferradini N, Staffolani P, Standardi A (1994) Effect of leaf excision time and age, BA concentration and dark treatment on in vitro shoot regeneration of M.26 apple rootstock. *J Hortic Sci* 69:679–685
- FAOSTAT (2005) Food and Agriculture Organization of the United Nations, <http://faostat.fao.org/default.aspx?alias=faostatclassic>
- Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. *Plant Cell Tissue Organ Cult* 16:75–87
- Fernandez-Maculet JC, Yang SF (1992) Extraction and partial characterization of the ethylene-forming enzyme from apple fruit. *Plant Physiol* 99:751–754
- Fernandez-Rivas M (2003) Cross-reactivity between fruit and vegetables. *Allergol Immunopathol* 31:141–146

- Fernandez-Rivas M, Cuevas M (1999) Peels of Rosaceae fruits have a higher allergenicity than pulps. *Clin Exp Allergy* 29:1239–1247
- Ferre J, Real MD, Vanrie J, Jansens S, Peferon M (1991) Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proc Natl Acad Sci USA* 88:5119–5123
- Ferree DC, Carlson RF (1987) Apple rootstocks. In: Rom RC, Carlson RF (eds) *Rootstocks for fruit crops*. John Wiley, New York, pp 107–143
- Fideghelli C, Sartoni A, Grasi F (2003) Fruit tree size and architecture. *Acta Hort* 622:279–293
- Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. *Bio/Technology* 5:807–813
- Fluhr R, Mattoo AK (1996) Ethylene: biosynthesis and perception. *Crit Rev Plant Sci* 15:479–523
- Forsline PL, Aldwinckle HS (2002) Natural occurrence of fire blight in USDA apple collection after 10 years of observation. *Acta Hort* 590:351–357
- Frey JE, Frey B, Sauer C, Kellerhals M (2004) Efficient low cost DNA-extraction and multiplex fluorescent PCR method for high-throughput marker-assisted selection (MAS) in apple breeding. *Plant Breed* 123:554–557
- Fritsch R, Ebner C, Kraft D (1997) Allergenic cross reactivities: pollens and vegetable foods. *Clin Rev Allergy Immunol* 15:397–404
- Gane R (1934) Production of ethylene by some ripening fruits. *Nature* 134:1008
- Gane R (1935) The formation of ethylene by plant tissue and its significance in the ripening of fruit. *J Pomol Hortic Sci* 13:351–358
- Gao Z, Maurousset L, Lemoine R, Yoo S-D, van Nocker S, Loeschner W (2003) Cloning, expression, and characterization of sorbitol transporters from developing sour cherry fruit and leaf sink tissues. *Plant Physiol* 131:1566–1575
- Gao Z, Jayanty S, Beaudry R, Loeschner W (2005) Sorbitol transporter expression in apple sink tissues: implications for fruit sugar accumulation and watercore development. *J Am Soc Hortic Sci* 130:261–268
- Gelvin SB (2003). *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Molec Biol Rev* 67:16–37
- Gianfranceschi L, Koller L, Seglias N, Kellerhals N, Gessler C (1996) Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. *Theor Appl Genet* 93:199–204
- Gianfranceschi L, Seglias N, Tarchini R, Komjanc M, Gessler C (1998) Simple sequence repeats for the genetic analysis of apple. *Theor Appl Genet* 96:1069–1076
- Good X, Kellogg JA, Wagoner W, Langhoffs D, Matsumura W, Bestwick RK (1994) Reduced ethylene synthesis by transgenic tomatoes expressing S-adenosylmethionine hydrolase. *Plant Mol Biol* 26:781–790
- Gould F (1998) Sustaining the efficacy of Bt toxins. In: Hardy RWF, JB Segelken (eds) *Agricultural biotechnology and environmental quality: gene escape and pest resistance*. National Agricultural Biotechnology Council (NABC), Ithaca, New York, Report 10, pp 77–86
- Grant CR, Ap Rees T (1981) Sorbitol metabolism by apple seedlings. *Phytochemistry* 20:1505–1511
- Gygax M, Gianfranceschi L, Liebhard R, Kellerhals M, Gessler C, Patocchi A (2004) Molecular markers linked to the apple scab resistance gene Vbj derived from *Malus baccata jackii*. *Theor Appl Genet* 109:1702–1709
- Hamilton AJ, Lycett GW, Grierson D (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature* 346:284–287
- Hamilton AJ, Bouzayen M, Grierson D (1991) Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. *Proc Natl Acad Sci USA* 88:7434–7437
- Hammerschlag FA, Zimmerman RH, Yadava UL, Hunsucker S, Gercheva P (1997) Effect of antibiotics and exposure to an acidified medium on the elimination of *Agrobacterium tumefaciens* from apple leaf explants and on shoot regeneration. *J Am Soc Hortic Sci* 122:758–763
- Hanke V, Norelli JL, Aldwinckle HS (1999) Transformation of apple cultivars with T4-lysozyme gene to increase fire blight resistance. *Acta Hort* 489:253–256

- Hanke V, Hiller I, Klotzsche G, Winkler K, Egerer J, Richter K, Norelli JL, Aldwinckle HS, Geibel M, Fischer M, Fischer C (2000) Transformation in apple for increased disease resistance. *Acta Hort* 538:611–616
- Hanke V, Kim WS, Geider K, Hale C, Mitchell R (2002) Plant transformation for induction of fire blight resistance: transgenic apples expressing viral EPS-depolymerase. *Acta Hort* 590:393–395
- Hemmat M, Weeden NF, Aldwinckle HS, Brown SK (1998) Molecular markers for the scab resistance (Vf) region in apple. *J Am Soc Hortic Sci* 123:992–996
- Hemmat M, Brown SK, Weeden NF (2002) Tagging and mapping scab resistance genes from R122740-7A apple. *J Am Soc Hortic Sci* 127:365–370
- Hemmat M, Brown SK, Aldwinckle HS, Weeden NF (2003) Identification and mapping of markers for resistance to apple scab from 'Antonovka' and 'Hansen's baccata #2'. *Acta Hort* 622:153–161
- Hirai M (1981) Purification and characteristics of sorbitol-6-phosphate dehydrogenase from loquat leaves. *Plant Physiol* 67:221–224
- Hirai M (1983) Seasonal changes in sorbitol-6-phosphate dehydrogenase in loquat leaf. *Plant Cell Physiol* 24:925–931
- Hofmann C, Luthy P, Hutter R, Pliska V (1988a) Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *Eur J Biochem* 173:85–91
- Hofmann C, Vanderbruggen H, Hofte H, Van Rie J, Jansens S, Van Mellaert H (1988b) Specificity of *Bacillus thuringiensis*  $\delta$ -endotoxin is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc Natl Acad Sci USA* 85:7844–7848
- Hoffmann-Sommergruber K (2002) Pathogenesis-related (PR) proteins identified as allergens. *Biochem Soc Trans* 30:930–935
- Hoffmann-Sommergruber K (2005) The SAFE project: 'Plant food allergies: field to table strategies for reducing their incidence in Europe', an EC-funded study. *Allergy* 60:436–442
- Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* 53:242–255
- Holefors A, Zhongtian X, Welander M (1998) Transformation of the apple rootstock M26 with the *rolA* gene and its influence on growth. *Plant Sci* 136:69–78
- Hsieh LS, Moos M, Lin Y (1995) Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening. *J Allergy Clin Immunol* 96:960–970
- Huang F, Buschman LL, Higgins RA, McGaughey WH (1999) Inheritance of resistance to *Bacillus thuringiensis* toxin (Dispel ES) in the European corn borer. *Science* 284:965–967
- Huang P-L, Parks JE, Rottmann WH, Theologies A (1991) Two genes encoding 1-aminocyclopropane-1-carboxylate synthesis in zucchini (*Cucurbita pepo*) are clustered and similar but differentially regulated. *Proc Natl Acad Sci USA* 88:7021–7025
- Hulme AC, Rhodes MJC, Galliard T, Wolltorton LSC (1968) Metabolic changes in excised fruit tissue. IV. Changes occurring in discs of apple peel during the development of the respiration climacteric. *Plant Physiol* 43:1154–1161
- Hulme AC (1971) The biochemistry of fruits and their products, vol 2. Academic Press, London, pp 333–373
- Hunt MD, Eannetta NT, Yu H, Newman SM, Steffens JC (1993) cDNA cloning and expression of potato polyphenol oxidase. *Plant Mol Biol* 21:59–68
- Jackson M (1997) Hormones from roots as signals for the shoots of stressed plants. *Trends Plant Sci* 2:22–28
- Jain SM, Isihii K (2003) Micropropagation of woody trees and fruits. Kluwer, London
- Jakubowski T, Zagaja SW (2000) 45 years of apple rootstock breeding in Poland. *Acta Hort* 538:723–727
- James DJ (1987) Cell and tissue culture technology for the genetic manipulation of temperate fruit trees. *Biotechnol Genet Eng Rev* 5:33–79

- James DJ (1999) A simple and reliable protocol for the detection of apple stem grooving virus by RT-PCR and in a multiplex PCR assay. *J Virol Methods* 83:1–9
- James DJ (2001) Long term assessment of the effects of in vitro chemotherapy as a tool for *Apple stem grooving virus* elimination. *Acta Hort* 550:459–462
- James DJ, Dandekar AM (1991) Regeneration and transformation of apple (*Malus pumila* Mill.). In: Lindsey K (ed) *Plant tissue culture manual: fundamentals and applications*. Kluwer, Dordrecht, pp 1–18
- James DJ, Passey AJ, Rugini E (1988) Factors affecting high frequency plant regeneration from apple leaf tissues cultured in vitro. *J Plant Physiol* 132:148–154
- James DJ, Passey AJ, Barbara DJ, Bevan MW (1989) Genetic transformation of apple (*Malus pumila* Mill.) using a disarmed Ti-binary vector. *Plant Cell Rep* 7:658–661
- James DJ, Passey AJ, Barbara DJ (1990) Regeneration and transformation of apple and strawberry using disarmed Ti-binary vectors. *Acta Hort* 280:495–502
- James DJ, Uratsu SL, Cheng J, Negri P, Viss P, Dandekar AM (1993) Conditions that induce *Agrobacterium Vir* genes also enhance apple cell transformation. *Plant Cell Rep* 12:559–563
- James DJ, Passey AJ, Barker SA (1994) Stable gene expression in transgenic apple tree tissues and segregation of transgenes in the progeny – preliminary evidence. *Euphytica* 77:119–121
- James DJ, Passey AJ, Baker SA, Wilson FM (1996) Transgenes display stable patterns of expression in apple fruit and Mendelian segregation in the progeny. *Bio/Technology* 14:56–60
- James DJ, Trytten PA, MacKenzie GH, Towers N, French CJ (1997) Elimination of apple stem grooving virus by chemotherapy and development of an immunocapture RT-PCR for rapid sensitive screening. *Annu Appl Biol* 131:459–470
- Janik J, Cummins JN, Brown SK, Hemmat M (1996) Apples. In: Janik J, Moore JN (eds) *Fruit breeding*. John Wiley, New York, pp 61–63
- Janssens GA, Goderis IJ, Broekaert WF, Broothaerts W (1995) A molecular method for S-allele identification in apple based on allele-specific PCR. *Theor Appl Genet* 91:691–698
- Kader AA, Norelli JL, Aldwinckle HS, Bauer DW, Beer SV, Momol MT, Saygili H (1999) Evaluation of the *hrpN* gene for increasing resistance to fire blight in transgenic apple. *Acta Hort* 489:247–250
- Kader JC (1996) Lipid-transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:627–654
- Kanamaru N, Ito Y, Komori S, Saito M, Kato H, Takahashi S, Omura M, Soejima J, Shitake K, Yamada K, Yamaki S (2004) Transgenic apple transformed by sorbitol-6-phosphate dehydrogenase cDNA. Switch between sorbitol and sucrose supply due to its gene expression. *Plant Sci* 167:55–61
- Kanayama Y, Yamaki S (1993) Purification and properties of NADP-dependent sorbitol-6-phosphate dehydrogenase from apple seedlings. *Plant Cell Physiol* 34:819–823
- Kanayama Y, Yamaki S (1994) Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit. *Plant Cell Physiol* 35:887–892
- Kanayama Y, Mori H, Imaseki H, Yamaki S (1992) Nucleotide sequence of a cDNA encoding NADP-sorbitol-6-phosphate dehydrogenase from apple. *Plant Physiol* 100:1607–1608
- Kanayama Y, Sakanishi K, Mori H, Yamaki S (1996) Expression of the gene for NADP-dependent sorbitol-6-phosphate dehydrogenase in apple seedlings. *Plant Cell Physiol* 36:1139–1141
- Karesová R, Paprstein F (2001) Apple chlorotic leaf spot virus in germplasm collection of fruit species. *Acta Hort* 550:250–264
- Kellerhals M, Mertschinger K, Gessler C (2004) Use of genetic resources in apple breeding and for sustainable fruit production. *J Fruit Ornamental Plant Res* 12:54–62
- Khanizadeh S, Groleau Y, Granger R, Cousineau J, Rousselle GL (2000) New hardy rootstocks from the Quebec apple breeding program. *Acta Hort* 538:719–721
- Kidd F, West C (1922) Report of the Food Investigation Board, London. 1921. Great Britain Department of Science and Industry Research, Food Investigation Board, London, pp 14–16
- Kidd F, West C (1925) The course of respiratory activity throughout the life of an apple. Great Britain Department of Science and Industry Research, Food Investigation Board, London, pp 27–33

- Kidd F, West C (1933) The effects of ethylene and apple vapours on the ripening of fruits. Great Britain Department of Science and Industry Research, Food Investigation Board, London, pp 55–58
- Kieber JJ, Ecker JR (1993) Ethylene gas: it's not just for ripening any more. *Trends Genet* 9:356–362
- Kim JY, Seo YS, Kim JE, Sung S-K, Song KJ, An G, Kim WT (2001) Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple. *Plant Sci* 161:1145–1152
- Kim JY, Lee J-R, Hong S-T, Yoo Y-K, An G, Kim WT (2003) Molecular cloning and analysis of anthocyanin biosynthesis genes preferentially expressed in apple skin. *Plant Sci* 165:403–413
- Kirby MJ, Guise CM, Adams AN (2001) Comparison of bioassays and laboratory assays for Apple stem grooving virus. *Acta Hort* 550:281–284
- Kitahara K, Matsumoto S (2002) Sequence of the S10 cDNA from 'McIntosh' apple and a PCR-digestion identification method. *HortScience* 37:187–190
- Kitahara K, Fukui H, Soejima J, Matsumoto S (1999) Cloning and sequencing of a new S-gene 'Sg-RNase' (accession no. AB019184) from *Malus domestica* Borkh. 'Indo'. *Plant Physiol* 119:1567
- Kitahara K, Soejima J, Komatsu H, Fukui H, Matsumoto S (2000) Complete sequences of the S-genes, Sd- and Sh-RNase cDNA in apple. *HortScience* 35:712–715
- Kitto SL (1997) Commercial micropropagation. *HortScience* 32:1012–1014
- Klee HJ, Hayford MB, Kretzmer KA, Barry GF, Kishore GM (1991) Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* 3:1187–1193
- Knowles BH, Ellar DJ (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis*  $\delta$ -endotoxins with different insect specificity. *Biochem Biophys Acta* 924:509–518
- Ko K, Norelli L, Brown SK, Aldwinckle HS, Doring K, Altman A, Ziv M, Izhar S (1999) Galaxy lines transgenic for *attacin* E and T4 lysozyme genes have increased resistance to fire blight. *Curr Plant Sci Biotechnol Agric* 36:507–511
- Ko KS, Norelli JL, Reynold JP, Aldwinckle HS, Brown SK (2002) T4 lysozyme and attacin genes enhance resistance of transgenic 'Galaxy' apple against *Erwinia amylovora*. *J Am Soc Hortic Sci* 127:515–519
- Koller B, Gianfranceschi L, Seglias N, McDermott J, Gessler C (1994) DNA markers linked to *Malus floribunda* 821 scab resistance. *Plant Mol Biol* 26:597–602
- Korvan SS, Skirvin RM (1984) Nomenclature of the cultivated apple. *HortScience* 19:177–180
- Krebitz M, Wagner B, Ferreira F, Peterbauer C, Campillo N, Witty M, Kolarich D, Steinkellner H, Scheiner O, Breiteneder H (2003) Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *J Mol Biol* 329:721–730
- Kundu JK (2001) To the diagnosis and distribution of the *Apple stem pitting virus* in the Czech Republic. *Acta Hort* 550:275–280
- Labuschagne IF, Louw JH, Schmidt K, Sandie A (2002) Genetic variation in chilling requirement in apple progeny. *J Am Soc Hortic Sci* 127:663–672
- Labuschagne IF, Louw JH, Schmidt K, Sandie A (2003) Selection for increased budbreak in apple. *J Am Soc Hortic Sci* 128:363–373
- Laimer M (2003) Detection and elimination of viruses and phytoplasmas from pome and stone fruit trees. *Hortic Rev* 28:187–236
- Laimer M, Mendoca D, Maghuly F, Marzhan G, Leopold S, Khan M, Balla I, Katinger H (2005) Review: biotechnology of temperate fruit trees and grapevines. *Acta Biochem Pol* 52(3):673–678
- Lambardi M, DeCarlo A (2003) Application of tissue culture to the germplasm conservation of the temperate broad-leaf trees. In: Jain SM, Ishii K (eds) *Micropropagation of woody trees and fruits*. Kluwer, Dordrecht, pp 815–840
- Lambert C, Tepfer D (1992) Use of *Agrobacterium rhizogenes* to create transgenic apple trees having an altered organogenic response to hormones. *Theor Appl Genet* 85:105–109



- Lancaster JE, Dougall DK (1992) Regulation of skin color in apples. *Crit Rev Plant Sci* 10:487–502
- Layee M, Knighton ML (1995) A full-length cDNA encoding 1-aminocyclopropane-1-carboxylate synthase from apple. *Plant Physiol* 107:1017–1018
- Lerch K (1981) Copper monooxygenases: tyrosinase and dopamine  $\beta$ -monooxygenase. In: Siegel H (ed) *Metal ions in biological systems*, vol 13. Marcel Dekker, New York, pp 143–186
- Lewis DH (1984) Occurrence and distribution of storage carbohydrates in vascular plants. In: Lewis DH (ed) *Storage carbohydrates in vascular plants*. Cambridge University Press, Cambridge, pp 1–52
- Liebhart R, Koller B, Patocchi A, Kellerhals M, Pfammatter W, Jermini M, Gessler C (2003) Mapping quantitative field resistance against apple scab in a 'Fiesta x Discovery' progeny. *Phytopathology* 93:493–501
- Litwinczuk W (2002) Propagation of black chokecherry (*Aronia melanocarpa* Elliot) through in vitro culture. <http://www.ejpau.media.pl>
- Lister CE, Lancaster JE, Walker JRL (1996) Phenylalanine ammonia-lyase (PAL) activity and its relationship to anthocyanin and flavonoid levels in New Zealand-grown apple cultivars. *J Am Soc Hortic Sci* 121:281–285
- Lister CE, Lancaster JE, Sutton KH, Walker JRL (1997) Aglycone and glycoside specificity of apple skin flavonoid glycosyltransferase. *J Sci Food Agric* 75:378–382
- Liu Q, Salih S, Hammerschlag F (1998) Etiolation of 'Royal Gala' apple (*Malus x domestica* Borkh.) shoots promotes high-frequency shoot organogenesis and enhanced  $\beta$ -glucuronidase expression from stem internodes. *Plant Cell Rep* 18:32–36
- Liu Q, Sun Q, Sun Y, Zhao H, Hammerschlag FA (1999) Transgenic 'Royal Gala' apple plants with cecropin-MB39 gene has increased resistance to *Erwinia amylovora*. China Association of Agricultural Science Societies, Research Progress in Plant Protection and Plant Nutrition. China Agricultural Press, Beijing, pp 48–56
- Liu Q, Ingersoll J, Owens L, Salih S, Meng R, Hammerschlag F (2001) Response of transgenic Royal Gala apple (*Malus x domestica* Borkh.) shoots carrying a modified cecropin MB39 gene, to *Erwinia amylovora*. *Plant Cell Rep* 20:306–312
- Loescher WH (1987) Physiology and metabolism of sugar alcohols in higher plants. *Physiol Plant* 70:533–557
- Loescher WH, Marlow GC, Kennedy RA (1982) Sorbitol metabolism and sink-source interconversions in developing apple leaves. *Plant Physiol* 70:335–339
- Lowenstein H, Eriksson NE (1983) Hypersensitivity to food among birch pollen-allergic patients. *Allergy* 138:577–587
- Luby JJ (2003) Taxonomic classification and brief history. In: Ferree DC, Warrington IJ (eds) *Apples: botany, production and uses*. CABI, Cambridge, pp 1–14
- Lucyszyn N, Quoirin M, Anjos A, Sierakowski M-R (2005) Blends of agar/galactomannan for Marubakaido apple rootstock shoot proliferation. *Polimeros Cienc Technol* 15:146–150
- Ma FW, Wang JC, Rong W (1990) Effects of plant growth regulator on in vitro propagation of apple cultivation. *J Fruit Sci* 7:201–206
- MacIntosh SC, Stone TB, Jokerst RS, Fuchs RL (1991) Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. *Proc Natl Acad Sci USA* 88:8930–8933
- Maheswaran G, Welander M, Hutchinson JE, Graham MW, Richards D (1992) Transformation of apple rootstock 'M26' with *Agrobacterium tumefaciens*. *J Plant Physiol* 39:560–568
- Maleck K, Lawton K (1998) Plant strategies for resistance to pathogens. *Curr Opin Biotechnol* 9:208–213
- Maliepaard C, Alston FH, van Arkel G, Brown LM, Chevreau E, Dunemann F, Evans KM, Gardiner S, Guilford P, van Heusden AW, Janse J, Laurens F, Lynn JR, Manganaris AG, den Nijs APM, Periam N, Rikkerink E, Roche P, Ryder C, Sansavini S, Schmidt H, Tartarini S, Verhaegh JJ, Veielink-van Ginkel M, King GJ (1998) Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. *Theor Appl Genet* 97:60–73
- Manganaris AG, Alston FH (1997) Genetics of superoxide dismutase in apple. *Theor Appl Genet* 95:484–489



- Martin GC, Miller AN, Castle LA, Morris JW, Morris RO, Dandekar AM (1990) Feasibility studies using  $\beta$ -glucuronidase as a gene fusion marker in apple, peach, and radish. *J Am Soc Hortic Sci* 115:689–691
- Marzban G, Pühringer H, Dey R, Brynda S, Martinelli A, Zaccarini M, Kolarich D, Altmann F, Katinger H, Laimer M (2005) Localization and distribution of major apple allergens in fruit tissue. *Plant Sci* 169:387–394
- Matsumoto S, Kitahara K (2000) Discovery of a new self-incompatibility allele in apple. *HortScience* 35:1329–1332
- Maximova SN, Dandekar AM, Guiltinan MJ (1998) Investigation of *Agrobacterium*-mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate limiting. *Plant Mol Biol* 37:549–559
- McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 14:269–276
- McGaughey WH (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science* 229:193–195
- McGaughey WH, Beeman RW (1988) Resistance to *Bacillus thuringiensis* in colonies of Indian meal moth and almond moth (Lepidoptera: Pyralidae). *J Econ Entomol* 81:28–33
- McGaughey WH, Johnson DE (1987) Toxicity of different serotypes and toxins of *Bacillus thuringiensis* to resistant and susceptible Indian meal moths (Lepidoptera: Pyralidae). *J Econ Entomol* 80:1122–1126
- McKeon T, Yang SF (1987) Biosynthesis and metabolism of ethylene. In: Davies PJ (ed) *Plant hormones and their role in plant growth and development*. Martinus Nijhoff, Dordrecht, pp 94–112
- Mehlenbacher SA (1995) Classical and molecular approaches to breeding fruit and nut crops for disease resistance. *HortScience* 30:466–477
- Mehra PN, Sachdeva S (1984) Embryogenesis in apple in vitro. *Phytomorphology* 34:26–36
- Mehta R, Handa A, Mattoo A (1997) Interactions of ethylene and polyamines in regulating fruit ripening. In: Kanellis AK, Chang C, Kende H, Grierson D (eds) *Biology and biotechnology of the plant hormone ethylene*. Kluwer, Dordrecht, pp 321–326
- Mehta R, Handa A, Mattoo AK (1999) Ethylene, polyamines and fruit ripening. In: Altman A, Ziv M, Izhar S (eds) *Plant biotechnology and in vitro biology in the 21st century*. Kluwer, Dordrecht, pp 591–595
- Menzel W, Zahn Y, Maiss E (2003) Multiplex RT-PCR-ELISA compared with bioassay for the detection of four apple viruses. *J Virol Methods* 110:153–157
- Meyer P, Heidmann I, Forkmann G, Saedler H (1987) A new petunia flower color generated by transformation of a mutant with a maize gene. *Nature* 330:677–678
- Mohan R, Soccol CR, Quoirin M, Pandey A (2004) Use of sugarcane bagasse as an alternative low cost support material during the rooting stage of apple micropropagation. *In Vitro Cell Dev Biol-Plant* 40:408–411
- Moing A, Langlois N, Svanella L, Zanetto A, Gaudillere JP (1997) Variability in sorbitol: sucrose ratio in mature leaves of different *Prunus* species. *J Am Soc Hortic Sci* 122:83–90
- Mol JNM, Stuitje AR, van der Krol A (1989) Genetic manipulation of floral pigmentation genes. *Plant Mol Biol* 13:287–294
- Morgan JM (1984) Osmoregulation and water stress in higher plants. *Annu Rev Plant Physiol* 35:299–319
- Morgante M, Hanafey M, Powell W (2002) Microsatellites are preferentially associated with non repetitive DNA in plant genomes. *Nat Genet* 30:194–200
- Mullis KF, Faloan F, Scharf S, Saiki R, Horn G (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol* 51:263–273
- Murashige T, Tucker DPH (1969) Growth factor requirements of citrus tissue culture. *Proc 1st Int Citrus Symp* 3:1155–1161

- Murata M, Nishimura M, Murai N, Haruta M, Homma S, Itoh Y (2001) A transgenic callus showing reduced polyphenol oxidase activity and lower browning potential. *Biosci Biotechnol Biochem* 65:383–388
- Narendar SN, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang S, Wilde HD, Kodrzycki RJ, Zhang C, Gause KC, Parks DW, Hinchey MA (2005) Forest biotechnology: innovative methods, emerging opportunities. *In Vitro Cell Dev Biol-Plant* 41:701–717
- Navarro L, Roistacher CN, Murashige T (1975) Improvement of shoot-tip grafting in vitro for virus-free citrus. *J Am Soc Hortic Sci* 100:471–479
- Negm FB, Loescher WH (1979) Detection and characterization of sorbitol dehydrogenase from apple callus tissue. *Plant Physiol* 64:69–73
- Negm FB, Loescher WH (1981) Characterization and partial purification of aldose-6-phosphate reductase (alditol-6-phosphate: NADP-1-oxidoreductase) from apple leaves. *Plant Physiol* 67:139–142
- Nemetchinov L, Hadidi A, Candresse T, Foster JA, Verdervskaya TD (1995) Sensitive detection of *Apple chlorotic leafspot virus* from infected apple or peach tissue using RT-PCR, IC-RT-PCR, or multiplex IC-RT-PCR. *Acta Hort* 386:51–62
- Newcomb RD, Crowhurst RN, Gleave AP, Rikkerink EHA, Allan AC, Beuning LL, Bowen JH, Gera E, Jamieson KR, Janssen BJ, Laing WA, McArtney S, Nain B, Ross GS, Snowden KC, Souleyre EJE, Walton EF, Yauk Y-K (2006) Analyses of expressed sequence tags from apple. *Plant Physiol* 141:147–166
- Noiraud N, Maurousset L, Lemoine R (2001) Identification of a mannitol transporter, AgMaT1, in celery phloem. *Plant Cell* 13:695–705
- Nolasco G, de Blas C, Torres V, Ponz F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. *J Virol Methods* 45:201–218
- Norelli JL, Aldwinckle HS (1993) The role of aminoglycoside antibiotics in the regeneration and selection of neomycin phosphotransferase-transgenic apple tissue. *J Am Soc Hortic Sci* 118:311–316
- Norelli JL, Aldwinckle HS, Destefano-Beltran L, Jaynes JM (1994) Transgenic 'Malling 26' apple expressing the attacin E gene has increased resistance to *Erwinia amylovora*. *Euphytica* 77:123–128
- Norelli JL, Mills J, Aldwinckle HS (1996) Leaf wounding increases efficiency of *Agrobacterium*-mediated transformation of apple. *HortScience* 3:1026–1027
- Norelli JL, Mills JZ, Jensen LA, Momol MT, Aldwinckle HS, Tobutt KRE, Alston FH (1999) Genetic engineering of apple for increased resistance to fire blight. *Acta Hort* 484:541–546
- Norelli JL, Borejsza Wysocka E, Reynoird JP, Aldwinckle HS, Geibel M, Fischer M, Fischer C (2000) Transgenic 'Royal Gala' apple expressing *attacin* E has increased field resistance to *Erwinia amylovora* (fire blight). *Acta Hort* 538:631–633
- Norelli JL, Holleran HT, Johnson WC, Robinson TL, Aldwinckle HS (2003) Resistance of Geneva and other apple rootstocks to *Erwinia amylovora*. *Plant Dis* 87:26–32
- Nosarszewski M, Clements AM, Downie AB, Archbold DD (2004) Sorbitol dehydrogenase expression and activity during apple fruit set and early development. *Physiol Plant* 121:391–398
- Oeller PW, Min-Wong L, Taylor LP, Pike DA, Theologis A (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* 254:437–439
- Oraguzie NC, Soejima J, Fukusawa-Akada T, Kudo K, Komatsu H, Kotoda N (2003) Apple breeding progress in Japan. *Acta Hort* 622:583–590
- Oraguzie NC, Tamamoto T, Soejima J, Susuki T, De Silva N (2005) DNA fingerprint of apple (*Malus* spp.) rootstocks using simple sequence repeats. *Plant Breed* 124:197–202
- Paek KY, Chakrabarty D, Hahn Plant Cell EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plants. *Plant Cell Tissue Organ Cult* 81:287–300
- Pan QY, Quebedeaux B (1995) Effects of elevated CO<sub>2</sub> on sorbitol partitioning in sink and source apple leaves. *Hortscience* 30:770
- Panis B, Lambardi M (2005) Status of cryopreservation technologies in plants (crops and forest trees): the role of biotechnology. *Villa Gualino, Turin*, pp 43–54

- Patocchi A, Bigler B, Koller B, Kellerhals M, Gessler C (2004) *Vr2*: a new apple scab resistance gene. *Theor Appl Genet* 109:1087–1092
- Paul H, Belaizi M, Sangwan-Norreel BS (1994) Somatic embryogenesis in apple. *J Plant Physiol* 143:78–86
- Paul H, Daigny G, Sangwan-Norreel BS (2000) Cryopreservation of apple (*Malus × domestica* Borkh.) shoot tips following encapsulation-dehydration or encapsulation-vitrification. *Plant Cell Rep* 19:768–774
- Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci USA* 88:3324–3328
- Phipps JB, Robertson KR, Smith PG, Rohrer JR (1990) A checklist of the subfamily Maloideae (Rosaceae). *Can J Bot* 68:2209–2269
- Pierik LLM (1991) Commercial micropropagation in Western Europe and Israel. In: Debergh PC, Zimmerman RH (eds) *Micropropagation: technology and application*. Kluwer, Dordrecht, pp155–166
- Predieri S, Fasolo F, Lalavasi F (1989) High frequency shoot regeneration from leaves of apple rootstock M.26 (*Malus pumila* Mill.). *Plant Cell Tissue Organ Cult* 17:133–142
- Pühringer H, Moll D, Hoffmann-Sommergruber K, Watillon B, Katinger H, Laimer da Câmara Machado M (2000) The promoter of an apple YPR10 gene, encoding the major apple allergen Mal d1, is stress and pathogen-inducible. *Plant Sci* 152:35–50
- Pühringer H, Zinöcker I, Marzbán G, Katinger H, Laimer M (2003) MdAP, a novel protein in apple, is associated with the major allergen Mal d 1. *Gene* 321:173–183
- Puite KJ, Schaart JG (1996) Genetic modification of the commercial apple cultivars Gala, Golden Delicious and Elstar via an *Agrobacterium tumefaciens*-mediated transformation method. *Plant Sci* 119:125–133
- Raese JT, Williams MW, Billingsley HD (1978) Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. *J Am Soc Hortic Sci* 103:796–801
- Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. *Curr Opin Plant Biol* 5:94–100
- Raffa K (1989) Genetic engineering of trees to enhance resistance to insects. *Bioscience* 39:524–534
- Ranney TG, Bassuk NL, Whitlow TH (1991) Osmotic adjustment and solute constituents in leaves and roots of water-stressed cherry (*Prunus*) trees. *J Am Soc Hortic Sci* 116:684–688
- Reid M (1987) Ethylene in plant growth and development and senescence. In: Davies PJ (ed) *Plant hormones and their role in plant growth and development*. Martinus Nijhoff, Dordrecht, pp 94–112
- Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. *Trends Plant Sci* 2:152–159
- Ridgwell RJ, Bielecki RL (1978) Sorbitol-1-phosphate and sorbitol-6-phosphate in apricot leaves. *Phytochemistry* 17:407–409
- Robinson T, Anderson L, Azarenko A, Barritt B, Baugher T, Brown G, Couvillon G, Cowgill W, Crassweller R, Domoto P, Embree C, Fennell A, Garcia E, Gaus A, Granger R, Greene G, Hirst P, Hoover E, Johnson S, Kushad M (2003) Performance of Cornell-Geneva apple rootstocks with 'Liberty' as the scion in NC-140 trials across North America. *Acta Hort* 622:521–530
- Ross GS, Knighton ML, Lay-Yee M (1992) An ethylene-related cDNA from ripening apples. *Plant Mol Biol* 19:231–238
- Roush RT (1998) Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? *Phil Trans R Soc Lond B* 353:1777–1786
- Rowhani A, Maningas MA, Lile LS, Daubert SD, Golino DA (1995) Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. *Phytopathology* 85:347–352

- Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491
- Sakai A (1960) Survival of the twigs of woody plants at  $-196^{\circ}\text{C}$ . *Nature* 185:393–394
- Sakai A (2000) Development of cryopreservation techniques. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm. International Plant Genetic Resources Institute, Rome, pp 1–7
- Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep* 9:30–33
- Sakanishi K, Kanayama Y, Mori M, Yamada K, Yamaki S (1998) Expression of the gene for NADP-dependent sorbitol-6-phosphate dehydrogenase in peach leaves of various developmental stages. *Plant Cell Physiol* 39:1372–1374
- Salcedo G, Diaz-Perales A, Sanchez-Monge R (1999) Fruit allergy: plant defence proteins as novel potential panallergens. *Clin Exp Allergy* 29:1158–1160
- Salcedo G, Sanchez-Monge R, Diaz-Perales A, Garcia-Casado G, Barberer D (2004) Review: plant non-specific lipid transfer proteins as food and pollen allergens. *Clin Exp Allergy* 34:1336–1341
- Sandanayaka WRM, Bus VGM, Connolly P, Newman R (2003) Characteristics associated with woolly apple aphid resistance, *Erwinia lanigerum*, of three apple rootstocks. *Entomol Exp Appl* 109:63–72
- Sassa H, Nishio T, Kowayama Y, Hirano H, Koba T, Ikehashi H (1996) Self-incompatibility (S) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. *Mol Gen Genet* 250:547–557
- Sato T, Theologis A (1989) Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene synthesis in plants. *Proc Natl Acad Sci USA* 86:6621–6625
- Saure MC (1990) External control of anthocyanin formation in apple. *Sci Hort* 42:181–218
- Schnepf E, Crickmore N, Van Rie J, Lerecus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticide crystal proteins. *Microbiol Mol Biol Rev* 62:775–806
- Schobert B (1977) Is there an osmotic regulatory mechanism in algae and higher plants? *J Theor Biol* 68:17–26
- Sherman TD, Vaughn KC, Duke SO (1991) A limited survey of the phylogenetic distribution of polyphenol oxidase. *Phytochemistry* 30:2499–2506
- Sherman WB, Beckman TG (2003) Climatic adaptation in fruit crops. *Acta Hort* 622:411–428
- Sims SR, Stone TB (1991) Genetic basis of tobacco budworm resistance to an engineered *Pseudomonas fluorescens* expressing the delta-endotoxin of *Bacillus thuringiensis kurstaki*. *J Inv Pathol* 57:206–210
- Spanu P, Reinhardt D, Boller T (1991) Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes. *EMBO J* 10:2007–2013
- Sriskandarajah S, Goodwin P (1998) Conditioning promotes regeneration and transformation in apple leaf explants. *Plant Cell Tissue Organ Cult* 53:1–11
- Sriskandarajah S, Goodwin PB, Speirs J (1994) Genetic transformation of apple scion cultivar ‘Delicious’ via *Agrobacterium tumefaciens*. *Plant Cell Tissue Organ Cult* 36:317–329
- Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG (1995) Molecular genetics of plant disease resistance. *Science* 268:661–667
- Steffens JC, Harel E, Hunt MD (1994) Polyphenol oxidase. In: Ellis BE (ed) Genetic engineering of plant secondary metabolism. Plenum Press, New York, pp 275–312
- Stone TB, Sims SR, Marrone PG (1989) Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. *J Invert Pathol* 53:228–234
- Stow J, Alston F, Hatfield S, Genge P (1993) New selections with inherently low ethylene production. *Acta Hort* 326:85–92

- Sucurani M, Piccioni E, Standardi A (2001) Micropropagation and preparation of synthetic seed in M.26 apple rootstocks I: attempts towards saving labor in the production of adventitious shoot tips suitable for encapsulation. *Plant Cell Tissue Organ Cult* 66:207–216
- Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T (1999) An allele of the ripening-specific 1-aminocyclopropane-1-carboxylic acid synthase gene (*ACS1*) in apple fruit with a long storage life. *Plant Physiol* 119:1297–1303
- Szankowski I, Briviva K, Fleschhut J, Schonherr J, Jacobsen H-J, Kiesecker H (2003) Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a PGIP gene from kiwi (*Actinidia deliciosa*). *Plant Cell Rep* 22:141–149
- Tabashnik BE, Cushing NL, Finson N, Johnson MW (1990) Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J Econ Entomol* 83:1671–1676
- Tabashnik BE, Finson N, Johnson MW (1991) Managing resistance to *Bacillus thuringiensis*: lessons from the diamondback moth (Lepidoptera: Plutellidae). *J Econ Entomol* 84:49–55
- Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* 259:508–510
- Tao R, Uratsu SL, Dandekar AM (1995) Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. *Plant Cell Physiol* 36:525–532
- Tao R, Dandekar AM, Uratsu SL, Vail PV, Tebbets JS (1997) Engineering genetic resistance against insects in Japanese persimmon using the *cryIA(c)* gene of *Bacillus thuringiensis*. *J Am Soc Hortic Sci* 122:764–771
- Tartarini S, Sansavini S (2003) Advances in the use of molecular markers in pome fruit breeding. *Acta Hort* 622:129–140
- Tartarini S, Gianfranceschi L, Sansavini S, Gessler C (1999) Development of reliable PCR markers for the selection of *Vf* gene conferring scab resistance in apple. *Plant Breed* 118:183–186
- Teo G, Suzuki Y, Uratsu SL, Lampinen B, Ormande N, Hu W, DeJong T, Dandekar AM (2006) Silencing leaf sorbitol synthesis alters long distance partitioning and apple fruit quality. *Proc Natl Acad Sci USA* (in press)
- Theologis T (1994) Control of ripening. *Curr Opin Biotechnol* 5:152–157
- Touster O, Shaw DRD (1962) Biochemistry of acyclic polyols. *Physiol Rev* 42:181–225
- Towill LE, Forsline PL, Walters C, Waddell JW, Laufmann J (2004) Cryopreservation of *Malus* germplasm using a winter vegetative bud method: results from 1915 accessions. *CryoLetters* 25:323–334
- Turner MC, Jones MM (1980) Turgor maintenance by osmotic adjustment: a review and evaluation. In: Turner NC, Krammer PJ (eds) *Adaptation of plants to water and high temperature stress*. Wiley-Interscience, John Wiley, New York, pp 87–103
- Tzfira T, Citovsky V (2002). Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends Cell Biol* 12:121–129
- US Apple Statistics (2005) Apple statistics. US Apple Association, <http://www.usapple.org/industry/applestats/index.cfm>
- Vaeck M, Reynaerts A, Hofte H, Jansens S, DeBeuckeleer M, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. *Nature* 328:33–37
- Vail PV, Tebbets JS, Hoffmann DF, Dandekar AM (1991) Response of production and postharvest walnut pests to *Bacillus thuringiensis* insecticidal crystal protein fragments. *Biol Cont* 1:329–333
- Van Der Straeten D, Van Wiemeersch L, Goodman H, Van Montagu M (1990) Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. *Proc Nat Acad Sci USA* 87:4859–4863
- Vanek-Krebitz M, Hoffman-Sommergrubere K, Laimer da Camara Machado M, Susani M, Ebner C, Kraft D, Scheider O, Breiteneder H (1995) Cloning and sequencing of Mad d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem Biophys Res Comm* 214:539–550

- Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol* 55:85–97
- Van Nerum I, Geerts M, Van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative 'new' S-alleles. *Theor Appl Genet* 103:584–591
- Van Rie J, McGaughey WH, Johnson DE, Barnett BD, Van Mellaert H (1990) Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* 247:72–74
- Verdoort L, Van Haute A, Goderis IJ, De Witte K, Keulemans J, Broothaerts W (1998) Use of the multiallelic self-incompatibility gene in apple to assess homozygosity in shoots obtained through haploid induction. *Theor Appl Genet* 96:294–300
- Vinatzer BA, Patocchi A, Tartarini S, Gianfranceschi L, Sansavini S, Gessler C (2004) Isolation of two microsatellite markers from BAC clones of the *Vf* scab resistance region and molecular characterization of scab resistant accessions in *Malus* germplasm. *Plant Breed* 123:321–326
- Walker JRL, Ferrar PH (1998) Diphenol oxidases, enzyme-catalysed browning and plant disease resistance. In: Tombs MP (ed) *Biotechnology and genetic engineering reviews*, vol 15. Intercept, Andover, pp 457–498
- Wang Z, Stutte GW (1992) The role of carbohydrates in active osmotic adjustment in apple under water stress. *J Am Soc Hortic Sci* 117:816–823
- Wang Z, Quebedeaux B, Stutte GW (1995) Osmotic adjustment – effect of water stress on carbohydrates in leaves, stems and roots of apple. *Aust J Plant Physiol* 22:747–754
- Wang Z, Quebedeaux B, Stutte GW (1996) Partitioning of (<sup>14</sup>C) glucose into sorbitol and other carbohydrates in apple under water stress. *Aust J Plant Physiol* 23:245–251
- Webb KL, Burley JWA (1962) Sorbitol translocation in apple. *Science* 137:766
- Webster T (2002) Dwarfing rootstocks: past, present and future. *Compact Fruit Tree* 35:67–72
- Webster T, Wertheim SJ (2003) Apple rootstocks. In: Ferree D, Warrington I (eds) *Apples: botany, production and uses*. CAB International, Wallingford, pp 91–124
- Welander M (1988) Plant regeneration from leaf and segments of shoots raised in vitro from mature apple trees. *J Plant Physiol* 132:738–744
- Wen-jun S, Forde B (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res* 17:83–85
- Wertheim SJ (1998) Apple rootstocks. In: Wertheim SJ (ed) *Rootstock guide*. Fruit Research Station, Wilhelminadorp, pp 19–60
- Wetzel T, Candresse T, Ravelonandro M, Dunez J (1991) A polymerase chain reaction assay adopted to plum pox virus detection. *J Virol Methods* 33:355–365
- Whetter JM, Taper CD (1966) Seasonal occurrence of sorbitol (D-glucitol) in buds and leaves of *Malus*. *Can J Bot* 41:175–177
- Whiteley HR, Schnepf HE (1986) The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annu Rev Microbiol* 40:549–576
- Williams EB, Kuc J (1969) Resistance in *Malus* to *Venturia inaequalis*. *Annu Rev Phytopathol* 7:223–246
- Wimmer R, Olsson M, Petersen MTN, Hatti-Kaul R, Petersen SB, Muller N (1997) Towards molecular understanding of protein stabilization: interaction between lysozyme and sorbitol. *J Biotechnol* 55:85–100
- Wu Q, Szakacs-Dobozi M, Hemmat M, Hrazdina G (1993) Endopolygalacturonase in apples (*Malus domestica*) and its expression during fruit ripening. *Plant Physiol* 102:219–225
- Yamada K, Oura Y, Mori H, Yamaki S (1998) Cloning of NAD-dependent sorbitol dehydrogenase from apple fruit and gene expression. *Plant Cell Physiol* 39:1375–1379
- Yamaguchi H, Kanayama Y, Yamaki S (1994) Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit. *Plant Cell Physiol* 35:887–892
- Yamaguchi H, Kanayama Y, Soejima J, Yamaki S (1996) Changes in the amounts of the NAD-dependent sorbitol dehydrogenase and its involvement in the development of apple fruit. *J Am Soc Hortic Sci* 121:848–852
- Yamaki S (1980) Property of sorbitol-6-phosphate dehydrogenase and its connection with sorbitol accumulation in apple. *HortScience* 15:268–270



- Yamaki S, Ishikawa K (1986) Roles of four sorbitol-related enzymes and invertase in the seasonal alterations of sugar metabolism in apple tissues. *J Am Soc Hortic Sci* 111:134–137
- Yao J, Cohen D, Atkinson R, Richardson K, Morris B (1995) Regeneration of transgenic plants from the commercial apple cultivar Royal Gala. *Plant Cell Rep* 14:407–412
- Yao J-L, Cohen D, van den Brink R, Morris B (1999) Assessment of expression and inheritance patterns of three transgenes with the aid of techniques for promoting rapid flowering of transgenic apple trees. *Plant Cell Rep* 18:727–732
- Yepes L, Aldwinckle HS (1994) Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell Tissue Organ Cult* 37:257–269
- Yip W-K, Dong J-G, Yang SF (1991) Purification and characterization of 1-aminocyclopropane-1-carboxylate synthase from apple fruits. *Plant Physiol* 95:251–257
- Yoshikawa N, Matsuda H, Oda Y, Isogai M, Takahashi T, Ito T, Yoshida K (2001) Genome heterogeneity of *Apple stem pitting virus* in apple trees. *Acta Hort* 550:285–290
- Zhang L-Y, Peng Y-B, Pelleschi-Travier P, Fan Y, Lu Y-F, Lu Y-M, Gao X-P, Shen Y-Y, Delrot S, Zhang D-P (2004) Evidence for apoplasmic phloem unloading in developing apple fruit. *Plant Physiol* 135:574–586
- Zhao Y, Wu Y, Engelmann F, Zhou M, Chen S (1999) Cryopreservation of apple in vitro shoot tips by the droplet freezing method. *CryoLetters* 20:109–112
- Zimmermann MH, Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH, Miburn JA (eds) *Transport of plants I: phloem transport*. Springer, Berlin Heidelberg New York, pp 480–502



## I.11 *Prunus* spp.

L. BURGOS<sup>1</sup>, C. PETRI<sup>1</sup>, and M.L. BADENES<sup>2</sup>

### 1 Introduction

The genus *Prunus*, belonging to the family Rosaceae, includes a large number of tree fruit species known as 'stone fruits' because the seed is encased within a hard, lignified stone-like endocarp. The edible portion of the fruit is the juicy mesocarp, although the genus also includes nut crop species such as almond (*P. dulcis* Miller). The major commercial stone fruit species are peach and nectarine (*P. persica* L.), European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), apricot (*P. armeniaca* L.) and the above mentioned almond. Most *Prunus* species used in commercial production represent only a small fraction of the genetic diversity available within the species. The western stone fruit cultivars, with a restricted germplasm base, but high fruit quality, productivity and superior handling characteristics, usually replace land race cultivars that are resistant to local biotic and abiotic stress factors (Scorza and Hammerschlag 1992).

### 2 Economic Importance

The genus *Prunus* has its centre of origin in western Asia. Commercial production for most species lies between latitudes 30 and 45° North and South. The plants are grown in all continents except Antarctica. Extremely cold temperatures below –35 to –40 °C or the absence of sufficient cold temperature to satisfy dormancy requirements are the major limiting factors for commercial stone fruit production. The world production of the five main species was estimated to be more than 31.6 million tons in 2004 (FAOSTAT, <http://apps.fao.org>). The world's leading producer of almond is the United States, followed by Iran, Spain and Italy. Apricot production is more evenly distributed among Turkey, Iran, Italy, France and Spain, with a total world production of almost 2.7 million tons. Turkey, Iran, the United States and Italy contribute 42% of the 1.9

<sup>1</sup> Departamento de Mejora de Frutales, CEBAS-CSIC, Apartado de Correos 164, 30.100 Murcia, Spain, e-mail: burgos@cebas.csic.es

<sup>2</sup> Departamento de Citricultura y Otros Frutales, IVIA, Apartado de Correos, Moncada, 46.113 Valencia, Spain

**Table 1.** World stone fruit production (in metric tons) in 2004, as indicated by continents and main producing countries (FAOSTAT database, <http://apps.fao.org>)

	Almonds	Apricots	Cherries	Peaches and nectarines	Plums
Africa	184,138	352,650	8,950	701,430	191,410
South Africa		68,000	950	210,000	39,000
Asia	396,510	1,290,700	653,250	7,599,342	5,378,272
China	22,000	83,000	15,000	5,782,000	4,384,000
Iran, Islamic Rep of	110,000	280,000	220,000	380,000	147,000
Japan			19,300	160,000	90,000
Europe	250,754	880,445	953,250	4,556,600	3,080,625
France	6,800	157,400	7,200	408,400	250,000
Greece	34,000	70,000	45,000	955,000	8,000
Italy	91,000	209,000	100,000	1,750,000	173,000
Spain	95,600	125,700	73,400	1,111,100	178,700
Turkey	38,000	440,000	255,000	460,000	205,000
North and Central America	818,160	89,979	228,660	1,681,283	767,162
United States	818,100	86,680	220,000	1,428,600	690,000
Oceania	10,000	24,242	10,266	134,309	28,697
Australia	10,000	19,742	9,460	126,983	26,547
South America	10,080	47,470	42,146	888,242	390,693
Argentina	480	25,000	6,800	272,442	127,413
Brazil				215,000	
Chile	9,600	22,000	33,000	275,000	240,000
Mexico	60	2,079	460	223,883	73,292
World production	1,669,642	2,685,486	1,896,522	15,561,206	9,836,859

million tons of the total cherry world production. More than 9.8 million tons of plums are produced worldwide, with China, as the leader, producing half of the output, followed by the United States, France, Chile, Turkey, Spain, Italy, Iran and Argentina. Peach and nectarine production, in excess of 15.5 million tons, accounts for almost the same production as the other four species together. The leading producers are China, Italy, the United States and Spain (Table 1).

### 3 Current Research and Development

Breeding research in *Prunus* has focused mainly on searching for individuals resistant to pests and diseases, control of plant growth, tolerance to abiotic stress, control of fruit ripening and dwarf plants for high density plantings. However, conventional breeding is constrained by the long juvenile periods of

temperate fruit trees, complex reproductive biology and high degree of heterozygosity. These drawbacks can be overcome by the use of a biotechnological approach, through which the fruit trees can be genetically transformed with genes of interest. Novel transgenic plants with the desired traits can be propagated vegetatively through tissue culture, providing unlimited production of the transgenic lines. Fixation through the sexual cycle is unnecessary and inconvenient if commercially accepted cultivars are genetically transformed. The development of molecular markers linked to the traits of interest is also important, as it allows desirable genotypes to be selected at early stages of plant development, saving cost and labor during transplanting, selecting and maintaining the adult trees.

### 3.1 Tissue Culture

Different techniques based on in vitro culture allow breeders to increase the efficiency of breeding programs and the selection of improved cultivars. Meristem culture has been used to obtain virus-free plant material (Kantha 1984) and to introduce and establish plant material in vitro (Pérez-Tornero et al. 1999). Somaclonal variation and in vitro selection have the advantage that they allow direct selection of novel phenotypes from large populations of physiologically uniform cells, under defined conditions, within a limited space and in a short period of time. Exploitation of somaclonal variation has facilitated the selection of peaches with increased resistance to bacterial infection (Hammerschlag and Ognjanov 1990), or tolerance to osmotic stress (Rajashekar et al. 1995). Embryo culture has been used to obtain early maturing *Prunus* cultivars (Ramming 1990). Also, major efforts are being devoted to develop efficient plant regeneration systems for *Prunus* species via either organogenesis or somatic embryogenesis, which are important for genetic transformation.

#### 3.1.1 Micropropagation

Micropropagation has been employed for commercial production of fruit and nut crops since the late 1970s. It was used initially for rootstocks for several tree fruits, especially peach. According to Murashige (1974), micropropagation comprises three steps that represent not only different processes of plant propagation, but also changes in the culture medium. Step 1 consists of the introduction and establishment of plant materials in vitro, where stem nodal segments have been most commonly used in *Prunus*. However, meristem tip culture has also been used in cases where internal contamination becomes problematic (Boxus and Quoirin 1974; Pérez-Tornero et al. 1999). Step 2 involves shoot multiplication and proliferation, while the aim of step 3 is to induce root formation from regenerated shoots. It has also been suggested to include step 0 for the preparation of the donor plants prior to in vitro culture (Debergh and Maene 1981) and step 4 for acclimatization of rooted shoots to

the external environment (George 1996).

Clonal propagation of various *Prunus* species in vitro has been described previously. These species include almonds (Tabachnik and Kester 1977; Rugini and Verma 1982; Channuntapipat et al. 2003), peaches and nectarines (Hammerschlag 1982, 1986; Hammerschlag et al. 1987), peach  $\times$  almond hybrids (Tabachnik and Kester 1977; Scorza et al. 1990; Marino and Ventura 1997), sweet and sour cherries (Snir 1982; Hammatt and Grant 1993, 1996; Pruski et al. 2005), plums (Druart 1992) and apricots (Snir 1984; Murai et al. 1997; Pérez-Tornero and Burgos 2000; Pérez-Tornero et al. 2000b). Most commercial production of micropropagated fruit trees has focused on the production of rootstocks. Studies on the behavior of trees grafted onto these tissue-cultured rootstocks revealed that grafting did not significantly influence fruit quality or yield (Zimmerman and Debergh 1991). Although grafting of scions onto seedling rootstocks is a common practice, the production of scions on their own roots has been limited. Nevertheless, the results of comparative studies have showed that own-rooted peach (Martin et al. 1983; Hammerschlag and Scorza 1991) and apricot (Pérez-Tornero et al. 2004) were found to fruit earlier and heavier than budded trees. Furthermore, micropropagated trees of 'Montmorency' sour cherry and 'Lambert' sweet cherry produced more yield than when grafted onto a commercial rootstock (Quamme and Brownlee 1993), but there was no difference in European plum (Webster and Wertheim 1993). These results suggest that own-rooted trees should be used when they are grafted onto seedlings, since it allows a better rooting system, as micropropagated trees usually consist of several well-developed roots.

Italy, Spain and Greece are the main European countries in which micropropagated *Prunus* has been commercialized. In Italy, total production in 2003 was more than 8 million rootstocks, 88% of which were produced by only three laboratories (Morini 2004). Among the rootstocks, the peach  $\times$  almond hybrid 'GF677' accounted for more than 50% of total production. A similar situation is found in Spain, where 7.5–8 million micropropagated plants, with more than 50% being the 'GF677' rootstock, are produced by four or five laboratories, one of which accounted for more than 80% of total production. 'GF677' is also the most common micropropagated rootstock in Greece, where about 2 million plants are produced annually (Pinochet, personal communication).

### 3.1.2 Embryo Culture

In vitro methods can be used to grow embryos that normally would abort. Embryo abortion may be due to incomplete development in early maturing cultivars or genetic incompatibility in interspecific hybrids. In these cases, the embryos generally are small and not developed enough for normal germination in soil. The culture of immature embryos from *Prunus* cultivars permits the use of early maturing cultivars as both seed parents and pollen parents in

breeding programs. Methodologies that improve the rescue of peach, plum and apricot embryos have been described previously (Ramming 1985; Burgos and Ledbetter 1993; Emershad and Ramming 1994; Ramming et al. 2003).

### 3.1.3 Adventitious Regeneration

The choice of appropriate explant is critical for morphogenesis in *Prunus* as it greatly affects the embryogenic or organogenic potential. The most commonly used explant is leaves prepared from cultured shoots (Laimer da Câmara Machado et al. 1988; Bassi and Cossio 1991; Hammatt and Grant 1998; Ainsley et al. 2000; Pérez-Tornero et al. 2000a). In addition to leaves, cotyledons (Mante et al. 1989), embryos (Schneider et al. 1992) and protoplasts (Ochatt and Power 1988) have been used. Generally, micropropagated shoots at stage 2 are most commonly employed as the source of explants, indicating that the physiological status of the explant may be important. Results of other studies indicate that regeneration can be improved by using explants derived from shoot cultures grown in the absence of or low concentrations of cytokinin during the last subculture (Escalettes and Dosba 1993; Miguel et al. 1996). The medium, where shoots used as explant donors are micropropagated, seems to exert a strong influence on the formation of adventitious buds (Burgos and Alburquerque 2003).

Explant age can influence regeneration rates. In general, young, actively growing tissues are more regenerative (Mante et al. 1989; Druart 1990; Miguel et al. 1996; Pérez-Tornero et al. 2000a). Other factors affecting regeneration include the carbon source (Declerck and Korban 1996; Nowak et al. 2004), growth regulators (Mante et al. 1989; Antonelli and Druart 1990; Goffreda et al. 1995; Hammatt and Grant 1998; Pérez-Tornero et al. 2000a), ethylene inhibitors and polyamines (Escalettes and Dosba 1993; Burgos and Alburquerque 2003; Petri et al. 2005), gelling agents (Pérez-Tornero et al. 2000a; Burgos and Alburquerque 2003) and culture conditions (Druart 1990; Miguel et al. 1996; Csányi et al. 1999; Pérez-Tornero et al. 2000a). Regeneration is also highly genotype-dependent, as summarized in Table 2. If genetic engineering is to be used as a tool for improvement of *Prunus* spp., it is imperative to develop a genotype-independent system for regeneration and transformation, based on meristematic cells with a high regeneration potential and/or using regeneration-promoting genes (Petri and Burgos 2005).

## 3.2 Transgenic Technology

Plant transformation is a process whereby DNA is introduced into plant cells and subsequently integrated into the plant genome. Transgenic plants can be produced by *Agrobacterium*-mediated transformation or direct gene transfer techniques, such as particle bombardment. However, these transformation methods rely on the availability of efficient tissue culture systems, in which

Table 2. Adventitious regeneration in *Prunus* spp.

Species	Cultivar/rootstock	Explant	Regeneration rate (%)	Regeneration efficiency <sup>a</sup> Shoots per explant	Reference
<i>P. armeniaca</i>	NJA82	Immature embryos	92	–	Goffreda et al. (1995)
	Zard		100	–	
	Bulda	Leaves	10	1–1.5	Pérez-Tornero
	Canino		50	1–1.5	et al. (2000a)
	Helena		30	1–1.5	
	Royal	Immature embryos	–	–	Pieterse 1989
<i>P. avium</i>	Sundrop	Cotyledons from immature embryos	100	–	Lane and Cossio (1986)
	H.152	Leaves	15	–	Escalettes and Dosba (1993)
	H.146		20	–	
	Van	Cotyledons from immature embryos	70	–	Lane and Cossio (1986)
	Burlat	Leaves	12	3.7	Tang et al. (2002)
	Hedelfinger		4	3.8	
	Napoleon		30	4.1	
	Schneiders		7	3.4	
	Pontavium	Calluses from mesophyll protoplasts <sup>c</sup>	12	5.0	Ochatt (1991)
	F12/1	Leaves	31	–	Hammatt and Grant (1998)
	Charger		47	–	
	Lapins	Leaves	71	–	Bhagwat and Lane (2004)
<i>P. avium</i> × <i>pseudocerasus</i>	Sweetheart		54	–	
	Colt	Stem internodal sections	22	–	James et al. (1984)
		Roots	–	–	Jones et al. (1984)
		Calluses from electroporated protoplasts	90	12.0	Ochatt et al. (1988)
		Protoplasts from root cell suspensions	40	5.9	Ochatt et al. (1987)

Table 2. (continued)

Species	Cultivar/rootstock	Explant	Regeneration rate (%)	Shoots per explant	Reference
<i>P. avium</i> x <i>sargentii</i>	1	Leaves	33	–	Hammatt and Grant (1998)
	16		9	–	
<i>P. canescens</i>	GM79	Leaves	29	3.4	Druart (1990)
<i>P. cerasifera</i>	P2980	Calluses from mesophyll protoplasts <sup>c</sup>	59	7.0	Ochatt (1992)
	CAB4D	Calluses from root protoplasts <sup>c</sup>	30	3.0	Ochatt (1990)
	CAB5H	Calluses from mesophyll protoplasts <sup>c</sup>	50	–	Ochatt and Power (1988)
	CAB4D	Roots from protoplast calluses	40	4.9	
	Morellenfeuer	Leaves	6	3.1	Tang et al. (2002)
	Beutal Spacher Rexelle		41	4.9	
<i>P. domestica</i>	Montmorency	Cotyledons from immature seeds	58	9.4	Mante et al. (1989)
	Besztercei	Leaves	32	–	Csányi et al. (1999)
	Bluefre	Leaves	25	2.0	Bassi and Cossio (1991)
	Susina di Dro		17	2.3	
	Węgierka Zwykla	Leaves	63	10.0	Nowak et al. (2004)
<i>P. dulcis</i>	Stanley	Hypocotyls	63	6.8	Mante et al. (1991)
	B70173	Cotyledons from immature seeds	85	18.7	Mante et al. (1989)
	Boa Casta	Leaves from in vitro shoots	29	2.2	Miguel et al. (1996)
		Leaves from germinated seeds	40	2.7	
		Leaves	44	3.4	Ainsley et al. (2000)
	Ne Plus Ultra		5	4.0	
	Nonpareil				
	Ne Plus Ultra	Cotyledons from immature seeds	93	16.0	Ainsley et al. (2001)
	Nonpareil		80	9.3	
	Carmel		100	11.5	
	Parkinson	Cotyledons from immature seeds	93	13.5	Ainsley et al. (2001)



Table 2. (continued)

Species	Cultivar/rootstock	Explant	Regeneration rate (%)	Shoots per explant	Regeneration efficiency <sup>a</sup>	Reference
<i>P. fruticosa</i> × <i>cerasus</i>	Black Eagle	Leaves	60	2.5		Dolgov (1999)
<i>P. persica</i>	Suncrest	Cotyledons from immature seeds	67	15.5		Mante et al. (1989)
	Loring		67	5.8		
	Belle		50	9.4		
	Boone County		60	4.8		
	Nemared	Cotyledons	18	1.0–5.0		Pooler and Scorza (1995)
	Nemaguard		5	1.0–5.0		
<i>P. serotina</i>	Flordaguard	Leaves	50	1.0–5.0		
	P16CL5		23	–		Gentile et al. (2002)
	Babygold 6		13	–		
	842 Standard		28	–		
	San Giorgio	Embryogenic callus from immature embryos	17	–		
	Yumyeong		22	–		
	– <sup>b</sup>		10	1.0		Dosba et al. (1991)
	PSB	Leaves	24	–		Hammatt and Grant (1998)
	2322		78	–		
	2339		8	–		
<i>P. spinosa</i>	Pavia E	Calluses from mesophyll protoplasts <sup>c</sup>	45	–		
	P51		72	15.0		Ochatt (1992)

<sup>a</sup> When regeneration rates or number of shoots per regenerating explant are not indicated, it has not been possible to deduce these from the information supplied by the authors in the corresponding paper

<sup>b</sup> Authors did not indicate the cultivar used as a seed source

<sup>c</sup> Regeneration rates correspond to the percentage of microcalli derived from protoplasts that regenerated adventitious shoots

shoots or plants can be regenerated from cultured cells or tissues via either organogenesis or somatic embryogenesis. In *Prunus*, the efficiency of plant regeneration of most species is generally low or genotype-dependent. This is due, at least in part, to the lack of understanding of basic developmental processes that underlie organogenesis and somatic embryogenesis. Utilization of tissue culture systems for genetic transformation ultimately must depend on an improved understanding and control of developmental mechanisms.

Genetic transformation allows discrete alteration of one or more traits in existing crop cultivars if an efficient tissue culture system is available. Transgenic plants have been used as a tool to analyze individual traits through the identification of the corresponding genes and to study their regulation and expression. Understanding gene regulation at the cellular and whole plant level, and identifying and evaluating agriculturally useful genes, are also possible. This has been carried out routinely in several species, such as tobacco, tomato, petunia and *Arabidopsis*. To date, a number of genetically modified plants have been cultivated worldwide. The total area of cultivation (1.7 million ha in 1996) has increased 40-fold to 67.7 million ha in 2003. This represents an estimated market value of \$4500 million or 15% of the total production (James 2003). However, transgenic *Prunus* plants are not presently commercialized.

### 3.2.1 *Agrobacterium*-Mediated Transformation

Our current understanding of the molecular mechanisms involved in T-DNA transfer and integration into the host plant genome has been reviewed recently (Gelvin 2000, 2003; Valentine 2003). It has been well documented that removal of all the genes within the T-DNA does not impede the transfer of T-DNA from *Agrobacterium* to the plant cell, but prevents tumor formation.

The virulence of the *Agrobacterium* strain varies with plant species (Cervera et al. 1998), and virulence can be stimulated by the presence of additional copies of the *virG* gene (Ghorbel et al. 2001). In *Prunus*, variation in bacterial virulence is also influenced by genotype (Dolgov and Firsov 1999; Miguel and Oliveira 1999; Ainsley et al. 2002; Yancheva et al. 2002). Several environmental factors, including pH, temperature and osmotic stress, have been shown to affect *vir* gene expression (Alt-Mörbe et al. 1989). Stachel et al. (1985) reported that the addition of the phenolic compound acetosyringone (3', 5'-dimethoxy-4' hydroxyacetophenone) to the culture medium also stimulated transcription of virulence genes in *Agrobacterium*. Similar stimulatory effect of acetosyringone on bacterial virulence has been observed in apricot (Laimer da Câmara Machado et al. 1992; Petri et al. 2004) and almond (Miguel and Oliveira 1999), but there was little effect in plum (Mante et al. 1991). *Prunus* transformation can also be affected by the duration of co-cultivation of inoculated explants with *Agrobacterium*. In general, the transformation frequency is increased with prolonged co-cultivation, but a period longer than 3–4 days can cause problems of *Agrobacterium* overgrowth.

### 3.2.2 Selectable Markers

Various selectable markers have been used during plant transformation to differentiate and select for a very small proportion of transformed cells from the majority of their non-transformed counterparts. To date, about 50 different selection systems have been reported, but only a few of them have practical application. A recent review identified antibiotics, such as kanamycin and hygromycin, and the herbicide phosphinothricin as the most commonly used selection systems (Miki and McHugh 2004). There is a concern that the transformation efficiencies are suboptimal with toxic substrates, because dying non-transformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells. Transformation of antibiotic-sensitive species may be improved by using non-toxic substrates and a 'positive' selection system (Joersbo 2001). In addition, reporter genes have often been used during transformation together with selectable markers. They have been used to confirm transgenic events where escapes may be common.

Over-expression of regeneration-promoting genes may be a useful selection system as only transformed, but not non-transformed cells, can be regenerated into plants in the absence of growth regulators. The *ipt* gene from *Agrobacterium* that encodes isopentenyl transferase, a key enzyme of cytokinin biosynthesis, is a classical example of a regeneration-promoting gene. Constitutive expression of *ipt* can adversely affect plant growth and development. This can be prevented by placing the gene under the control of an inducible promoter (Kunkel et al. 1999) or in an MAT (multi-autonomous transformation) vector, leading to its elimination from the transgenic plants (Ebinuma et al. 1997). We found that transformation of apricot with an MAT vector containing an *ipt* gene could notably improve the transformation efficiency (López-Noguera et al. 2006) compared to the standard transformation procedures used in our laboratory (Petri et al. 2006). Apart from *ipt*, information regarding other regeneration-promoting genes has been virtually lacking. Major efforts are being devoted to identify these genes, whose translation products may be associated with cytokinin synthesis and its recognition, or involved in promoting the vegetative-to-embryogenic or organogenic transition (Zuo et al. 2002).

### 3.2.3 Selection of Transformed Plants

Selection of transformed regenerants is a critical step in plant transformation. Antibiotics have been used most commonly as selection agents after integration of genes that confer antibiotic resistance. The concentration of the selective agent and timing of application must be optimized for each plant species. In *Prunus*, a progressive selection pressure has been used to recover transformed plants (Miguel and Oliveira 1999; Yancheva et al. 2002; Petri et al. 2006). Once putative transformed plants are obtained, it is important to confirm stable

integration of the introduced gene(s) and its expression in these plants and their progeny. In annual plants, this can be achieved by backcrossing or selfing the plant to determine whether the introduced gene is heritable. However, such an approach is not practical to perennial species because of their long generation time. Instead, transformation is usually confirmed by Southern blot analysis of the transformed tissue (Bhat and Srinivasan 2002). Depending on the information required, a variety of combinations of restriction enzymes and probes can be used in Southern analysis. Information on the number of loci at which the transgene is integrated, identification of independent transgenic plants by unique hybridization patterns, occurrence of truncation, rearrangements or multiple insertions at the same loci may be gained from the appropriate selection of enzymes and probes (Bhat and Srinivasan 2002).

### 3.3 Molecular Genetics

Recent advances in the genetics and genomics of *Prunus* were based on the development of molecular markers. These markers allow the construction of linkage maps that include the position of important traits. The construction of BAC libraries and the development of ESTs (expressed sequence tags), plus their mapping in an integrated physical map, are the most important features in *Prunus* genetics. Exploration of the potential of these new tools may lead to a better understanding of the biochemistry and genetics of *Prunus* species.

#### 3.3.1 Development of Molecular Markers

Restriction fragment length polymorphism (RFLP) was the first marker system developed (Beckman and Soller 1983). It was used in peach and almond to construct a genetic linkage map (Viruel et al. 1995; Abbott et al. 1998). Random amplified polymorphic DNA (RAPDs) (Williams et al. 1990) has been used in studies of diversity and the construction of linkage maps in *Prunus* (Chaparro et al. 1994; Hurtado et al. 2002). Other methods used in *Prunus* to investigate diversity and construct linkage maps involved simple sequence repeats (SSRs) (Tautz 1989; Morgante and Olivieri 1993) and amplified fragment length polymorphism (AFLPs) (Vos et al. 1995). Amongst the different techniques, SSRs and microsatellites are the most common techniques used. SSRs have proven to be highly polymorphic, easily reproducible and codominant markers. These features overcome some of the limitations of other marker systems such as dominance in RAPDs and AFLPs, the low reproducibility of RAPDs and low polymorphism of RFLPs. In addition, they are abundant and well distributed in the genome (Decroocq et al. 2003).

Primers flanking regions with SSRs have been developed in peach (Sosinski et al. 2000; Testolin et al. 2000; Yamamoto et al. 2001; Aranzana et al. 2002a, b; Dirlwanger et al. 2002), cherry (Downey and Iezzoni 2000; Cantini et al. 2001), apricot (Lopes et al. 2002; Hagen et al. 2004; Messina et al. 2004) and

Japanese plum (Mnejja et al. 2004). Conservation of flanking regions amongst taxa has been reported (Rosseto et al. 2002; Decroocq et al. 2003) and cross species amplification has been tested in *Prunus* (Cipriani et al. 1999; Downey and Iezzoni 2000; Sosinski et al. 2000; Dirlewanger et al. 2002). Consequently, there are a large number of primer flanking SSR sequences available for genetic studies. This molecular system has been used most recently in genetic diversity studies and the construction of linkage maps.

### 3.3.2 Linkage Maps in *Prunus*: Consensus Linkage Mapping and Traits Mapped

A reference map with 562 markers covering 519 cM was constructed in *Prunus* (Dirlewanger et al. 2004), adding the markers to a map reported previously (Joobeur et al. 1998). Thirteen maps constructed with a subset of these markers have allowed genome comparison amongst seven diploid ( $x = 8$ ) *Prunus* species. Comparing the positions of anchor markers (RFLPs, SSRs and isozymes) of the *Prunus* reference map with those other 13 maps, constructed with different *Prunus* populations, has shown that the genomes of the diploid species are essentially collinear, which is a great advantage for our comprehensive understanding of *Prunus* genetics (Dirlewanger et al. 2004).

### 3.3.3 Marker-Assisted Selection: the Mapping of Major Genes

Some important agronomic characters in fruit trees behave as major gene-inherited traits. These include disease resistance and vegetative flower, fruit and nut quality traits. Their simple inheritance makes them obvious targets for researching tightly linked markers for early selection. This is particularly true of traits that require complex analysis, for instance, analysis of resistance to diseases. Mapping of resistance genes can overcome these long procedures by an early selection assisted by molecular markers. Resistance genes have been mapped in apricot (Soriano et al. 2005) and peach (Decroocq et al. 2005). Other traits that cannot be evaluated until the plant has reached the adult stage, such as fruit characters or the self-incompatibility genotype, are also targets for molecular assisted selection. Table 3 summarizes the linkage maps available in *Prunus* species and those traits that have been mapped.

### 3.3.4 EST Sequencing, Database and Physical Map

Peach has been proposed as the model plant for genetics and genomics studies in the family Rosaceae. The reasons for this are the low genome size, the knowledge in genetics and the molecular biology resources available. Efforts are being made worldwide to develop genome-genetic resources for this species. In addition to marker development and the construction of linkage maps, large insert genomic libraries have been constructed for peach (Wang et al. 2001; Georgi et al. 2002), apricot (Vilanova et al. 2003a) and plum (Claverie et al. 2004).

**Table 3.** Linkage maps available in *Prunus* species and traits mapped. Adapted from Dirlewanger et al. (2004)

Linkage group	Trait	Species	Reference
G1	Fruit flesh color	Peach	Bliss et al. (2002)
	Sharka resistance	Apricot	Vilanova et al. (2003b)
	Ever growing	Peach	Wang et al. (2001)
	Flower color	Almond × peach	Jauregui (1998)
G2	Root-knot nematode resistance	Peach	Bliss et al. (2002); Yamamoto et al. (2001); Claverie et al. (2004) Lu et al. (1998)
	Shell hardness	Almond	Arús et al. (1998)
	Growth habit	Peach	Scorza et al. (2002)
	Double flowers	Peach	Chaparro et al. (1994)
G3	Flesh color around stone	Peach	Yamamoto et al. (2001)
	Anther color	Almond × peach	Joobeur (1998)
	Polycarpel	Peach	Bliss et al. (2002)
	Flower color	Peach	Yamamoto et al. (2001)
G4	Blooming time	Almond	Ballester et al. (2001)
	Flesh adhesion	Peach	Dettori et al. (2001); Yamamoto et al. (2001)
G5	Non acid fruit	Peach	Dirlewanger et al. (1998, 1999); Etienne et al. (2002)
	Kernel taste	Almond	Bliss et al. (2002)
	Skin hairiness	Peach	Dirlewanger et al. (1998, 1999); Bliss et al. (2002)
G6	Leaf shape	Peach	Yamamoto et al. (2001)
	Dwarf/normal	Peach	Yamamoto et al. (2001)
	Male sterility	Peach	Dirlewanger et al. (1998)
	Fruit shape	Peach	Dirlewanger et al. (1998, 1999)
	Self-incompatibility	Almond	Bliss et al. (2002)
G6–G8		Apricot	Ballester et al. (1998); Vilanova et al. (2003b)
	Fruit skin color	Peach	Yamamoto et al. (2001)
	Leaf color	Peach	Jauregui (1998); Yamamoto et al. (2001); Claverie et al. (2004)
G7	Resistance to powdery mildew	Peach	Dettori et al. (2001)
	Leaf gland		
	Root-knot nematode resistance	Myrabolan plum	Lecouls et al. (1999); Claverie et al. (2004)

A physical map for peach has been constructed using two BAC libraries and the RFLPs on the *Prunus* reference map as anchored loci (Zhebentyayeva et al. 2004). A database that integrates the anchored peach physical map and an extensive peach EST database are available at [www.genome.clemson.edu](http://www.genome.clemson.edu). Additional collections of ESTs from peach, almond and apricot are being released and are available on this website.

Markers tightly linked to important traits, quantitative character dissection using linkage maps, candidate gene approaches and genomic tools, such as the physical map and the map position of a large collection of ESTs are the main features of the progress made in *Prunus* in the last decade. Improved understanding of *Prunus* genetics based on the resources recently developed may allow the use of genomic technologies, already applied in annual species, to *Prunus* plants, hastening the progress of tree genetics.

## 4 Practical Applications of Transgenic Plants

Table 4 summarizes the successful incorporation of transgenes into *Prunus* species. Although the highest transformation frequency of 30% has been reported with the use of juvenile explants (Mante et al. 1991), the frequency was generally low, ranging from 0.2% (Miguel and Oliveira 1999) to 12.3% (Costa et al. 2006). In most transformation events with *Prunus* species, juvenile material was used as explants and only marker genes were introduced. Successful transformation using adult material has been reported in almond (Ramesh et al. 2006) and apricot cultivars (López-Noguera et al. 2006; Petri et al. 2006) as well as cherry cultivars (Song and Sink 2006) and rootstocks (da Câmara Machado et al. 1995; Druart et al. 1998; Gutiérrez-Pesce et al. 1998; Song and Sink 2006). Transgenic plants of apricot (Laimer da Câmara Machado et al. 1992) and plum (Ravelonandro et al. 1997) expressing the coat protein gene of plum pox virus were shown to confer sharka resistance. In cherry rootstocks, shoots transformed using *A. rhizogenes* displayed improved rooting (Gutiérrez-Pesce et al. 1998) and Basta resistance (Druart et al. 1998). Transformation of peaches using a 'shooty mutant' *A. tumefaciens* strain resulted in increased branching and reduced rooting (Smigocki and Hammerschlag 1991) (Table 4).

## 5 Conclusions and Future Challenges

Conventional breeding of *Prunus* has been constrained by the long reproductive cycle of the genus, with an extended juvenile growth phase, complex reproductive biology and high degree of heterozygosity. Although improved cultivars of most of the *Prunus* species have been obtained through conventional breeding, new technologies have the potential to reduce the time for cultivar



Table 4. Transformation of *Prunus* spp.

Species	Cultivar	Technique	Plasmid (strain)	Genes	Explant	TE (%) <sup>a</sup>	Reference
<i>P. armeniaca</i>	Kecksemeter	<i>A. tumefaciens</i>	pBinGUSint (LBA 4404)	<i>nptII</i> , <i>gus</i>	Cotyledons	-	Laimer da Câmara Machado et al. (1992)
	H152 (Screara × Stark Early Orange)	<i>A. rhizogenes</i>	pRi (A4) pRipMarcel35PpVcP (C7)	ADN-T ( <i>ipt</i> ) ADN-T ( <i>ipt</i> ), <i>ppvCp</i>	Shoots	-	Escalettes et al. (1994)
		<i>A. tumefaciens</i>	pBin19NPTII-GUSint (C58G) pGANPTII-PPVCp-GUS-HYG (C58C)	<i>nptII</i> , <i>gus</i> <i>nptII</i> , <i>gus</i> , <i>ppvCp</i> , <i>hyg</i>	Leaves	0	
	Helena	<i>A. tumefaciens</i>	pBIN19-sGFP (EHA105) pEXMGFP1 (EHA105)	<i>nptII</i> , <i>gfp</i> <i>nptII</i> , <i>gus</i> , <i>ipt</i>	Leaves Leaves	- 8.5	Petri et al. (2006) López-Noguera et al. (2006)
<i>P. avium</i>	Mazzard	<i>A. rhizogenes</i>	pRi1855 (NCPBP 1855)	ADN-T ( <i>ipt</i> )	Shoots <sup>b</sup>	0	Gutiérrez-Pesce et al. (1998)
<i>P. avium</i> × <i>pseudocerasus</i>	Colt	<i>A. rhizogenes</i>		ADN-T ( <i>ipt</i> )	Shoots <sup>b</sup>	2.5 <sup>c</sup>	
<i>P. cerasus</i>	Montmorency	<i>A. tumefaciens</i>	pBISN1 (EHA105)	<i>nptII</i> , <i>gus</i>	Leaves	3.1 3.3	Song and Sink (2006)
<i>P. cerasus</i> × <i>canescens</i>	Gisela 6						
<i>P. dawnykensis</i>	Damil	<i>A. rhizogenes</i> Biolistica	pRi 15834 + pBin19 'Basta' ATCC pUC 18 <i>basta-gus</i>	ADN-T ( <i>ipt</i> ), <i>bar</i> <i>gus</i> , <i>bar</i>	Shoots <sup>b</sup> Meristems	- -	Druart et al. (1998)
<i>P. domestica</i>	Stanley	<i>A. tumefaciens</i>	pCGN 7001, pCGN 7314 (EHA 101)	<i>nptII</i> , <i>gus</i>	Hypocotyls	10-30	Mante et al. (1991)
	Damas de Toulouse	<i>A. rhizogenes</i>	pRi (A4) pRipMarcel35PPVCp (C7)	ADN-T ( <i>ipt</i> ) ADN-T ( <i>ipt</i> ), <i>ppvCp</i>	Shoots <sup>b</sup>	0	Escalettes et al. (1994)
	Marianna (GF8-1)	<i>A. tumefaciens</i>	pBin19NPTII-GUSint (C58G) pGANPTII-PPVCp-GUS-HYG (C58C)	<i>nptII</i> , <i>gus</i> <i>nptII</i> , <i>gus</i> , <i>ppvCp</i> , <i>hyg</i>	Leaves	-	Escalettes et al. (1994)

Table 4. (continued)

Species	Cultivar	Technique	Plasmid (strain)	Genes	Explant	TE (%) <sup>a</sup>	Reference
<i>P. domestica</i>	Stanley	<i>A. tumefaciens</i>	pGA482GG/PPV-CP33 (C58 / Z707, EHA 101) pGA482GG/CPPRV (C58 / Z707)	<i>nptII, gus,</i> <i>ppvCp</i>	Hypocotyls	1.2	Scorza et al. (1994)
				<i>nptII, gus, prvCp</i>		2	Scorza
<i>nptII, gus, prvCp</i>	B70146	<i>A. tumefaciens</i>	pGA482GG/CPPRV				(C58 / Z707)
	Hypocotyls 3	3	et al. (1995)				
	Quetsche Kyustendilska sinya	<i>A. tumefaciens</i>	psmGFP (LBA 4404)	<i>nptII, gfp</i>	Leaves	0.8 2.7	Yancheva et al. (2002)
<i>P. dulces</i>	Supernova MN51	<i>A. tumefaciens</i>	pBinGUSint (LBA 4404)	<i>nptII, gus</i>	Leaves	0	Archilletti et al. (1995)
	Boa Casta (Clon VII)	<i>A. tumefaciens</i>	pFAJ3003, p35SGUSint (EHA 105)	<i>nptII, gus</i>	Leaves from germinated seeds	0.2	Miguel and Oliveira (1999)
	Nonpareil	<i>A. tumefaciens</i>	p35SGUSint (LBA 4404, EHA 105)	<i>nptII, gus</i>	Leaves	12.3	Costa et al. (2006)
	Ne Plus Ultra			<i>nptII, gfp</i> <i>manA</i>		0 0 5.6 6.8	Ainsley et al. (2002) Ramesh et al. (2006)
<i>P. fruticosa</i> × <i>avium</i>	Black Eagle	<i>A. tumefaciens</i>	pPCV635 (GV3101) p35SGUSint (EHA 105)	<i>afp, hpt</i> <i>nptII, gus</i>	Leaves	-	Dolgov (1999)
						-	Dolgov and Firsov (1999)
<i>P. incisa</i> × <i>serrula</i>	Inmil	<i>A. tumefaciens</i>	p35SGUSint (LBA 4404)	<i>nptII, gus</i>	Embryogenic calluses	-	Druart et al. (1998)
		<i>A. rhizogenes</i>	pRi 15834 + pBin19 'Basta' (ATCC)	ADN-T ( <i>ipt</i> ), <i>bar</i>	Shoots <sup>b</sup>	-	

Table 4. (continued)

Species	Cultivar	Technique	Plasmid (strain)	Genes	Explant	TE (%) <sup>a</sup>	Reference
<i>P. persica</i>	14DR60 Tennessee Natural PER 2D	<i>A. tumefaciens</i>	pCA472 (A281)	<i>nptII</i>	Embryogenic calluses Leaves	0	Scorza et al. (1990)
	Redhaven	<i>A. tumefaciens</i>	pTiA6 (tms328::Tn5) “Shooty mutant”	ADN-T ( <i>ipt</i> )	Immature embryos Shoots	0	Hammerschlag et al. (1989)
					Immature embryos	-	Smigocki and Hammerschlag (1991)
	Lovell	Biolística	pBI505, pBI426	<i>nptII</i> , <i>gus</i>	Embryo axes, immature embryos, embryo calluses, cotyledons, leaves and shoot apex	0	Ye et al. (1994)
<i>P. subhirtella</i>	Miraflores	<i>A. tumefaciens</i>	pBin19-sgfp	<i>nptII</i> , <i>gfp</i>	Seed embryo sections	3.6	Pérez-Clemente et al. (2004)
	Autumno rosa	<i>A. tumefaciens</i>	pBinGUSint (LBA 4404)	<i>nptII</i> , <i>gus</i>	Embryogenic calluses from leaf petioles	-	da Câmara Machado et al. (1995)

<sup>a</sup> Transformation efficiency. When not indicated, it could not be deduced from the information provided by the authors  
<sup>b</sup> These shoots produced transgenic roots from which transformed shoots were regenerated  
<sup>c</sup> Efficiency refers to the number of roots formed after *A. rhizogenes* infection

development and offer alternative breeding strategies that are not available to breeders. Progress has been made in the areas of regeneration, *Agrobacterium*-mediated transformation, gene isolation and mapping, but several obstacles remain to be overcome. This is especially true for the development of a genotype-independent system for tissue culture and genetic transformation, which may be achieved by the transformation of meristematic cells with a high regeneration potential and/or the use of regeneration-promoting genes. Also, the constraint should be addressed that European laws will allow neither the deliberate release of plants carrying antibiotic resistance genes after 2004, nor their commercialization after 2008 (Directive 2001/18/EEC of the European Parliament and the Council of the European Union). The development of a selectable marker-free transformation system for *Prunus* spp. is therefore a priority in future studies.

## References

- Abbott AG, Rajapakse S, Sosinski B, Lu ZX, Sossey-Alaoui K, Gannavarapu M, Reighard G, Ballard RE, Baird WV, Scorza R, Callahan A (1998) Construction of saturated linkage maps of peach crosses segregating for characters controlling fruit quality, tree architecture and pest resistance. *Acta Hort* 465:41–50
- Ainsley PJ, Collins GG, Sedgley M (2000) Adventitious shoot regeneration from leaf explants of almond (*Prunus dulcis* Mill.). *In Vitro Cell Dev Biol-Plant* 36:470–474
- Ainsley PJ, Hammerschlag FA, Bertozzi T, Collins GG, Sedgley M (2001) Regeneration of almond from immature seed cotyledons. *Plant Cell Tissue Organ Cult* 67:221–226
- Ainsley PJ, Collins GG, Sedgley M (2002) Factors affecting *Agrobacterium*-mediated gene transfer and the selection of transgenic calli in paper shell almond (*Prunus dulcis* Mill.). *J Hort Sci Biotechnol* 76:522–528
- Alt-Mörbe J, Kühlmann H, Schröder J (1989) Differences in induction of Ti plasmid virulence genes *virG* and *virD* and continued control of *virD* expression by four external factors. *Mol Plant-Microbe Interact* 2:301–308
- Antonelli M, Druart P (1990) The use of a brief 2,4-D treatment to induce leaf regeneration on *Prunus canescens* Bois. *Acta Hort* 280:45–50
- Aranzana MJ, García-Mas J, Carbó J, Arús P (2002a) Development and variability of microsatellite markers in peach. *Plant Breed* 121:87–92
- Aranzana MJ, Pineda A, Cosson P, Ascasibar J, Dirlewanger E, Cipriani G, Ryder CD, Testolin R, Abbott A, King GJ, Iezzoni AF, Arús P (2002b) A set of simple-sequence repeat (SSR) markers covering the *Prunus* genome. *Theor Appl Genet* 106:819–825
- Archillett T, Lauri P, Damiano C (1995) *Agrobacterium*-mediated transformation of almond leaf pieces. *Plant Cell Rep* 14:267–272
- Arús P, Ballester J, Jáuregui B, Joobeur T, Truco M, Vicente MC (1998) The European *Prunus* mapping project: update on marker development in almond. *Acta Hort* 484:331–338
- Ballester J, Boskovic R, Batlle I, Arús P, Vargas F, de Vicente MC (1998) Location of the self-incompatibility gene on the almond linkage map. *Plant Breed* 117:69–72
- Ballester J, Socías i Company R, Arús P, de Vicente MC (2001) Genetic mapping of a major gene delaying blooming time in almond. *Plant Breed* 120:268–270
- Bassi G, Cossio F (1991) In vitro shoot regeneration on “Bluefre” and “Susina di Dro” prune cultivars (*Prunus domestica* L.). *Acta Hort* 289:81–82
- Beckmann JS, Soller M (1983) Restriction fragment length polymorphisms in genetic improvement – methodologies, mapping and costs. *Theor Appl Genet* 67:35–43

- Bhagwat B, Lane WD (2004) In vitro regeneration from leaves of sweet cherry (*Prunus avium*) 'Lapins' and 'Sweetheart'. *Plant Cell Tissue Organ Cult* 78:173–181
- Bhat SR, Srinivasan S (2002) Molecular and genetic analysis of transgenic plants: considerations and approaches. *Plant Sci* 163:673–681
- Bliss FA, Arulsekaran S, Foolad MR, Becerra V, Gillen AM, Warburton ML, Dandekar AM, Kocsisne GM, Mydin KK (2002) An expanded genetic linkage map of *Prunus* based on an interspecific cross between almond and peach. *Genome* 45:520–529
- Boxus P, Quoirin M (1974) La culture de meristemes apicaux de quelques especes de *Prunus*. *Bull Soc R Bot Belg* 107:91–101
- Burgos L, Alburquerque N (2003) Low kanamycin concentration and ethylene inhibitors improve adventitious regeneration from apricot leaves. *Plant Cell Rep* 21:1167–1174
- Burgos L, Ledbetter CA (1993) Improved efficiency in apricot breeding: effects of embryo development and nutrient media on in vitro germination and seedling establishment. *Plant Cell Tissue Organ Cult* 35:217–222
- Cantini C, Iezzoni AF, Lombay WF, Boristki M, Struss D (2001) DNA fingerprinting of tetraploid cherry germplasms using simple sequence repeat. *J Am Soc Hortic Sci* 126:205–209
- Cervera M, López MM, Navarro L, Peña L (1998) Virulence and supervirulence of *Agrobacterium tumefaciens* in woody fruit plants. *Physiol Mol Plant P* 52:67–78
- Channuntapipat C, Sedgley M, Collins GG (2003) Micropropagation of almond cultivars Nonpareil and Ne Plus Ultra and the hybrid rootstock Titan × Nemaguard. *Sci Hort* 98:473–484
- Chaparro JX, Werner DJ, O'Malley DO, Sederoff RR (1994) Targeted mapping and linkage analysis of morphological isozyme, and RAPD markers in peach. *Theor Appl Genet* 87:805–815
- Cipriani G, Lot G, Huang W-G, Marrazzo MT, Peterlunger E, Testolin R (1999) AC/GT and AG/CT microsatellite repeats in peach (*Prunus persica* (L.) Batsch): isolation, characterization and cross-species amplification in *Prunus*. *Theor Appl Genet* 99:65–72
- Claverie M, Bosselut N, Lecouls AC, Voisin R, Lafargue B, Poizat C, Kleinhentz M, Laigret F, Dirlewanger E, Esmenjaud D (2004). Location of independent root-knot nematode resistance genes in plum and peach. *Theor Appl Genet* 108:765–773
- Costa MS, Miguel C, Oliveira MM (2006) An improved selection strategy and the use of acetosyringone in shoot induction medium increase almond transformation efficiency by 100-fold. *Plant Cell Tiss Organ* 85:205–209
- Csányi M, Wittner A, Nagy A, Balla I, Vértessy J, Palkovics L, Balázs E (1999) Tissue culture of stone fruit plants: basis for their genetic engineering. *J Plant Biotechnol* 1:91–95
- da Câmara Machado A, Puschmann M, Puhlinger H, Kremen R, Katinger HWD, Laimer da Câmara Machado M (1995) Somatic embryogenesis of *Prunus subhirtella autumnno rosa* and regeneration of transgenic plants after *Agrobacterium*-mediated transformation. *Plant Cell Rep* 14:335–340
- Debergh PC, Maene LJ (1981) A scheme for commercial propagation of ornamental plants by tissue culture. *Sci Hort* 14:335–345
- Declerck V, Korban SS (1996) Influence of growth regulators and carbon sources on callus induction, growth and morphogenesis from leaf tissues of peach (*Prunus persica* L Batsch). *J Hortic Sci* 71:49–55
- Decroocq V, Favé MG, Hagen LS, Bordenave L, Decroocq S (2003) Development and transferability of apricot and grape microsatellites across taxa. *Theor Appl Genet* 106:912–922
- Decroocq V, Fouligne M, Lambert P, Gall L, Mantin C, Pascal T, Schurdi-Levraud V, Kervella J (2005) Analogues of virus resistance genes map to QTLs for resistance to sharka disease in *Prunus davidiana*. *Mol Gen Genomics* 272:680–689
- Dettoni MT, Quarta R, Verde I (2001) A peach linkage map integrating RFLPs, SSRs, RAPDs, and morphological markers. *Genome* 44:783–790
- Dirlewanger E, Pronier V, Parvery C, Rothan C, Guye A, Monet R (1998) A genetic linkage map of peach (*Prunus persica* L. Batsch) using morphological, RFLP, isoenzyme, RAPD and AFLP markers. *Theor Appl Genet* 97:888–895

- Dirlewanger E, Moing A, Rothan C, Svanella L, Pronier V, Guye A, Plomion C, Monet R (1999) Mapping QTLs controlling fruit quality in peach *Prunus persica* (L.) Batsch. Theor Appl Genet 98:18–31
- Dirlewanger E, Cosson P, Tavaud M, Aranzana MJ, Poizat C, Zanetto A, Arús P, Laigret F (2002) Development of microsatellite markers in peach [*Prunus persica* (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry (*Prunus avium* L.). Theor Appl Genet 105:127–138
- Dirlewanger E, Graziano E, Joobeur T, Garriga-Caldere F, Cosson P, Howad W, Arus P (2004) Comparative mapping and marker-assisted selection in Rosaceae fruit crops. Proc Natl Acad Sci USA 101:9891–9896
- Dolgov SV (1999) Genetic transformation of sour cherry (*Cerasus vulgaris* Mill.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Transgenic trees, vol 44. Springer, Berlin Heidelberg New York, pp 29–38
- Dolgov SV, Firsov AP (1999) Regeneration and *Agrobacterium* transformation of sour cherry leaf discs. Acta Hort 484:577–579
- Dosba F, Massonie G, Maison P, Audergon JM (1991) Plum pox virus resistance of apricot. Acta Hort 293:569–579
- Downey SL, Iezzoni AF (2000) Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry, peach and sour cherry. J Am Soc Hortic Sci 125:76–80
- Druart P (1990) Effect of culture conditions and leaf selection on organogenesis of *Malus domestica* cv. McIntosh “Wijcik” and *Prunus canescens* Bois GM79. Acta Hort 280:117–124
- Druart P (1992) In vitro culture and micropropagation of plum (*Prunus* spp.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Transgenic trees, vol 44. Springer, Berlin Heidelberg New York, pp 279–303
- Druart P, Delporte F, Brazda M, Ugarte-Ballon C, da Câmara Machado A, Laimer da Câmara Machado M, Jacquemin J, Watillon B (1998) Genetic transformation of cherry trees. Acta Hort 468:71–76
- Ebinuma H, Sugita K, Matsunaga E, Yamakado M (1997) Selection of marker-free transgenic plants using the isopentenyl transferase gene. Proc Natl Acad Sci USA 94:2117–2121
- Emershad RL, Ramming DW (1994) Effects of media on embryo enlargement, germination and plant development in early-ripening genotypes of *Prunus* grown in vitro. Plant Cell Tissue Organ Cult 37:55–59
- Escalettes V, Dosba F (1993) In vitro adventitious shoot regeneration from leaves of *Prunus* spp. Plant Sci 90:201–209
- Escalettes V, Dahuron F, Ravelonandro M, Dosba F (1994) Utilisation de la transgénèse pour l'obtention de pruniers et d'abricotiers exprimant le gène de la protéine capsid du plum pox potyvirus. Bull OEPP/EPPO 24:705–711
- Etienne C, Rothan C, Moing A, Plomion C, Bodnes C, Svanella-Dumas L, Cosson P, Pronier V, Monet R, Dirlewanger E (2002) Candidate genes and QTLs for sugar and organic acid content in peach [*Prunus persica* (L.) Batsch]. Theor Appl Genet 105:145–159
- Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256
- Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. Microbiol Mol Biol R 67:16–37
- Gentile A, Monticelli S, Damiano C (2002) Adventitious shoot regeneration in peach [*Prunus persica* (L.) Batsch]. Plant Cell Rep 20:1011–1016
- George EF (1996) Plant propagation by tissue culture. Part 2: in practice. Exegetics, Edington
- Georgi L, Wang Y, Yvergniaux D, Ormsbee T, Inigo M, Reighard G, Abbott G (2002) Construction of a BAC library and its application to the identification of simple sequence repeats in peach [*Prunus persica* (L.) Batsch]. Theor Appl Genet 105:1151–1158
- Ghorbel R, La-Malfa S, López MM, Petit A, Navarro L, Peña L (2001) Additional copies of virG from pTiBo542 provide a super-transformation ability to *Agrobacterium tumefaciens* in citrus. Physiol Mol Plant P 58:103–110

- Goffreda JC, Scopel AL, Fiola JA (1995) Indole butyric acid induces regeneration of phenotypically normal apricot (*Prunus armeniaca* L.) plants from immature embryos. *Plant Growth Regul* 17:41–46
- Gutiérrez-Pesce P, Taylor K, Muleo R, Rugini E (1998) Somatic embryogenesis and shoot regeneration from transgenic roots of the cherry rootstock Colt (*Prunus avium* × *P. pseudocerasus*) mediated by pRi 1855 T-DNA of *Agrobacterium rhizogenes*. *Plant Cell Rep* 17:574–580
- Hagen LS, Chaib J, Fady B, Decroocq V, Bouchet P, Lambert P, Audergon JM (2004) Genomic and cDNA microsatellites from apricot (*Prunus armeniaca* L.). *Mol Ecol Notes* 4:724–745
- Hammatt N, Grant NJ (1993) Apparent rejuvenation of mature wild cherry (*Prunus avium* L.) during micropropagation. *J Plant Physiol* 141:341–346
- Hammatt N, Grant NJ (1996) Micropropagation of mature British wild cherry. *Plant Cell Tissue Organ Cult* 47:103–110
- Hammatt N, Grant NJ (1998) Shoot regeneration from leaves of *Prunus serotina* Ehrh. (black cherry) and *P. avium* L. (wild cherry). *Plant Cell Rep* 17:526–530
- Hammerschlag FA (1982) Factors affecting establishment and growth of peach shoots in vitro. *HortScience* 17:85–86
- Hammerschlag FA (1986) Peach. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry. Transgenic trees*, vol 44. Springer, Berlin Heidelberg New York, pp 170–193
- Hammerschlag FA, Ognjanov V (1990) Somaclonal variation in peach: screening for resistance to *Xanthomonas campestris* pv. *pruni* and *Pseudomonas syringae*. *Acta Hort* 280:408
- Hammerschlag FA, Scorza R (1991) Field performance of micropropagated, own-rooted peach trees. *J Am Soc Hortic Sci* 116:1089–1091
- Hammerschlag FA, Bauchan GR, Scorza R (1987) Factors influencing in vitro multiplication and rooting of peach cultivars. *Plant Cell Tissue Organ Cult* 8:235–242
- Hammerschlag FA, Owens LD, Smigocki AC (1989) *Agrobacterium*-mediated transformation of peach cells derived from mature plants that were propagated in vitro. *J Am Soc Hortic Sci* 114:508–510
- Hurtado MA, Vilanova S, Romero C, Abbott AG, Llácer G, Badenes ML (2002) Genetic linkage maps of two apricot cultivars (*Prunus armeniaca* L.) based on molecular markers. *Theor Appl Genet* 105:182–191
- James C (2003) Preview: global status of commercialized transgenic crops International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca. ISAAA Briefs 30:1–7
- James DJ, Passey AJ, Malhotra SB (1984) Organogenesis in callus derived from stem and leaf tissues of apple and cherry rootstocks. *Plant Cell Tissue Organ Cult* 3:333–341
- Jauregui B (1998) Localización de marcadores moleculares ligados a caracteres agronómicos en un cruce interespecífico de almendro × melocotonero. PhD Thesis, University of Barcelona
- Joersbo M (2001) Advances in the selection of transgenic plants using non-antibiotic marker genes. *Physiol Plant* 111:269–272
- Jones OP, Gayner JA, Watkins R (1984) Plant regeneration from callus tissue cultures of the cherry rootstock Colt (*Prunus avium* × *P. pseudocerasus*) and the apple rootstock M.25 (*Malus pumila*). *J Hortic Sci* 59:463–467
- Joobeur T, Viruel MA, de Vicente MC, Jauregui MB, Ballester J, Dettori MT, Verde I, Truco MJ, Messeguer R, Batlle I, Quarta R, Dirlewanger E, Arús P (1998) Construction of a saturated linkage map for *Prunus* using an almond × peach F<sub>2</sub> progeny. *Theor Appl Genet* 7:1034–1041
- Kartha KK (1984) Elimination of viruses. In: Vasil IK (ed) *Cell culture and somatic cell genetics of plants*. Academic Press, New York, pp 577–585
- Kunkel T, Niu QW, Chan YS, Chua NH (1999) Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. *Nat Biotechnol* 17:916–919
- Laimer da Câmara Machado M, da Câmara Machado A, Hanzer V, Mattanovich D, Himmler G, Katinger HWD (1988) Regeneration of shoots from leaf discs and stem microcuttings of fruit trees as a tool for transformation. *Acta Hort* 235:85–92
- Laimer da Câmara Machado M, da Câmara Machado A, Hanzer V, Weiss H, Regner F, Steinke-liner H, Mattanovich D, Plail R, Knapp E, Kalthoff B, Katinger HWD (1992) Regeneration of



- transgenic plants of *Prunus armeniaca* containing the coat protein gene of plum pox virus. *Plant Cell Rep* 11:25–29
- Lane WD, Cossio F (1986) Adventitious shoots from cotyledons of immature cherry and apricot embryos. *Can J Plant Sci* 66:953–959
- Lecouls AC, Rubio-Cabetas MJ, Minot JC, Voisin R, Bonnet A, Saleses G, Dirlwanger E, Esmejaud D (1999) RAPD and SCAR markers linked to the Ma1 root-knot nematode resistance gene in Myrabolan plum (*Prunus cerasifera* Ehr.). *Theor Appl Genet* 99:328–335
- Lopes MS, Sefc KM, Laimer M, Machado AC (2002) Identification of microsatellite loci in apricot. *Mol Ecol Notes* 2:24–26
- López-Noguera S, Petri C, Burgos L (2006) Production of marker-free transgenic plants after transformation of apricot cultivars. *Acta Hort* 717:225–227
- Lu ZX, Sosinski A, Reighard GL, Baird WV, Abbott AG (1998) Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. *Genome* 41:199–207
- Mante S, Scorza R, Cordts JM (1989) Plant regeneration from cotyledons of *Prunus persica*, *Prunus domestica*, and *Prunus cerasus*. *Plant Cell Tissue Organ Cult* 19:1–11
- Mante S, Morgens PH, Scorza R, Cordts JM, Callahan AM (1991) *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyl slices and regeneration of transgenic plants. *Bio/Technology* 9:853–857
- Marino G, Ventura M (1997) The influence of ethylene on in vitro rooting of GF677 (*Prunus persica* × *Prunus amygdalus*) hybrid peach rootstock. *In Vitro Cell Dev Biol-Plant* 33:26–29
- Martin C, Carré M, Vernoy R (1983) La multiplication végétative in vitro des végétaux ligneux cultivés: cas des arbres fruitiers et discussion générale. *Agronomie* 3:303–306
- Messina R, Lain O, Marrazzo MT, Cipriano G, Testolin R (2004) New set of microsatellite loci isolated in apricot. *Mol Ecol Notes* 4:432–434
- Miguel CM, Oliveira MM (1999) Transgenic almond (*Prunus dulcis* Mill.) plants obtained by *Agrobacterium* mediated transformation of leaf explants. *Plant Cell Rep* 18:387–393
- Miguel CM, Druart P, Oliveira MM (1996) Shoot regeneration from adventitious buds induced on juvenile and adult almond (*Prunus dulcis* Mill.) explants. *In Vitro Cell Dev Biol-Plant* 32:148–153
- Miki B, McHugh S (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J Biotechnol* 107:193–232
- Mnejja M, García-Más J, Howard W, Badenes ML, Arús P (2004) Simple-sequence repeat (SSR) markers of Japanese plum (*Prunus salicina* Lindl.) are highly polymorphic and transferable to peach and almond. *Mol Ecol Notes* 4:163–166
- Morgante M, Olivieri A (1993) PCR-amplified microsatellites as markers in plant genetics. *Plant J* 3:175–182
- Morini S (2004) Stato attuale della produzione di portinnesti mediante micropropagazione. *Frutticoltura* 12:33–36
- Murai Y, Harada H, Yamashita H (1997) In vitro propagation of apricot (*Prunus armeniaca* L.) cv. ‘Bakuoh junkyou’. *J Jpn Soc Hortic Sci* 66:475–480
- Murashige T (1974) Plant propagation through tissue cultures. *Annu Rev Plant Physiol* 25:135–166
- Nowak B, Miczynski K, Hudy L (2004) Sugar uptake and utilisation during adventitious bud differentiation on in vitro leaf explants of ‘Wegierka Zwykla’ plum (*Prunus domestica*). *Plant Cell Tissue Organ Cult* 76:255–260
- Ochatt SJ (1990) Plant regeneration from root callus protoplasts of sour cherry (*Prunus cerasus* L.). *Plant Cell Rep* 9:268–271
- Ochatt SJ (1991) Strategies for plant regeneration from mesophyll protoplast of the recalcitrant fruit and farmwoodland species *Prunus avium*. *Physiol Plant* 82:1:A15
- Ochatt SJ (1992) The development of protoplast-to-tree systems for *Prunus cerasifera* and *Prunus spinosa*. *Plant Sci* 81:253–259
- Ochatt SJ, Power JB (1988) An alternative approach to plant regeneration from protoplasts of sour cherry (*Prunus cerasus* L.). *Plant Sci* 56:75–79

- Ochatt SJ, Cocking EC, Power JB (1987) Isolation, culture and plant regeneration of colt cherry (*Prunus avium* × *pseudocerasus*) protoplasts. *Plant Sci* 50:139–143
- Ochatt SJ, Chand PK, Rech EL, Davey MR, Power JB (1988) Electroporation-mediated improvement of plant regeneration from colt cherry (*Prunus avium* × *pseudocerasus*) protoplasts. *Plant Sci* 54:165–169
- Pérez-Clemente RM, Pérez-Sanjuán A, García-Férriz L, Beltrán JP, Cañas LA (2004) Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an in vivo marker. *Mol Breed* 14:419–427
- Pérez-Tornero O, Burgos L (2000) Different media requirements for micropropagation of apricot cultivars. *Plant Cell Tissue Organ Cult* 63:133–141
- Pérez-Tornero O, Burgos L, Egea J (1999) Introduction and establishment of apricot in vitro through the regeneration of shoots from meristem tips. *In Vitro Cell Dev Biol-Plant* 35:249–253
- Pérez-Tornero O, Egea J, Vanoostende A, Burgos L (2000a) Assessment of factors affecting adventitious shoot regeneration from in vitro cultured leaves of apricot. *Plant Sci* 158:61–70
- Pérez-Tornero O, López JM, Egea J, Burgos L (2000b) Effect of basal media and growth regulators on the in vitro propagation of the apricot (*Prunus armeniaca*) cv. ‘Canino’. *J Hortic Sci Biotechnol* 75:283–286
- Pérez-Tornero O, López JM, Burgos L (2004) Variedades de albaricoquero autoenraizadas mediante cultivo in vitro: una alternativa de propagación al injerto. *Fruticultura Profesional* 144:41–44
- Petri C, Burgos L (2005) Transformation of fruit trees. Useful breeding tool or continued future prospect? *Transgenic Res* 14:15–26
- Petri C, Albuquerque N, García-Castillo S, Egea J, Burgos L (2004) Factors affecting gene transfer efficiency to apricot leaves during early *Agrobacterium*-mediated transformation steps. *J Hortic Sci Biotech* 79:704–712
- Petri C, Albuquerque N, Pérez-Tornero O, Burgos L (2005) Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and *Agrobacterium*-mediated transformation of leaf tissues. *Plant Cell Tissue Organ Cult* 82:105–111
- Petri C, López-Noguera S, Albuquerque N, Burgos L (2006) Regeneration of transformed apricot plants from leaves of a commercial cultivar. *Acta Hort* 717:233–235
- Pieterse RE (1989) Regeneration of plants from callus and embryos of ‘Royal’ apricot. *Plant Cell Tissue Organ Cult* 19:175–179
- Pooler MR, Scorza R (1995) Regeneration of peach [*Prunus persica* (L.) Batsch] rootstock cultivars from cotyledons of mature stored seed. *HortScience* 30:355–356
- Pruski K, Astatkie T, Nowak J (2005) Tissue culture propagation of Mongolian cherry (*Prunus fruticosa*) and Nanking cherry (*Prunus tormentosa*). *Plant Cell Tissue Org Cult* 82:207–211
- Quamme HA, Brownlee RT (1993) Early performance of micropropagated trees of several *Malus* and *Prunus* cultivars on their own roots. *Can J Plant Sci* 73:847–855
- Rajashekar G, Palmquist DE, Ledbetter CA (1995) In vitro screening procedure for osmotic tolerance in *Prunus*. *Plant Cell Tissue Organ Cult* 41:159–164
- Ramesh SA, Kaiser BN, Franks T, Collins G, Sedgley M (2006) Improved methods in *Agrobacterium*-mediated transformation of almond using positive (mannose/*pmi*) or negative (kanamycin resistance) selection-based protocols. *Plant Cell Rep* 25:821–828
- Ramming DW (1985) In ovule embryo culture of early-maturing *Prunus*. *HortScience* 20:419–420
- Ramming DW (1990) The use of embryo culture in fruit breeding. *HortScience* 25:393–398
- Ramming DW, Emershad RL, Foster C (2003) In vitro factors during ovule culture affect development and conversion of immature peach and nectarine embryos. *HortScience* 38:424–428
- Ravelonandro M, Scorza R, Bachelier JC, Labonne G, Levy L, Damsteegt VD, Callahan AM, Dunez J (1997) Resistance of transgenic *Prunus domestica* to plum pox virus infection. *Plant Dis* 81:1231–1235
- Rossetto M, McNally J, Henry RJ (2002) Evaluating the potential of SSR flanking regions for examining taxonomic relationships in the Vitaceae. *Theor Appl Genet* 104:61–66

- Rugini E, Verma DC (1982) Micropropagation of difficult-to-propagate almond (*Prunus amygdalus*, Batsch) cultivar. *Plant Sci Lett* 28:273–281
- Schneider KE, Speranzini D, Biggs AR (1992) Ontogeny of shoot regenerants on excised immature peach embryos. *Can J Plant Sci* 72:497–506
- Scorza R, Hammerschlag FA (1992) Stone fruits. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. University Press, Cambridge, pp 277–301
- Scorza R, Morgens PH, Cordts JM, Mante S, Callahan AM (1990) *Agrobacterium*-mediated transformation of peach (*Prunus persica* L. Batsch) leaf segments, immature embryos, and long-term embryogenic callus. *In Vitro Cell Dev Biol* 26:829–834
- Scorza R, Ravelonandro M, Callahan AM, Cordts JM, Fuchs M, Dunez J, Gonsalves D (1994) Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. *Plant Cell Rep* 14:18–22
- Scorza R, Levy L, Damsteegt VD, Yepes LM, Cordts JM, Hadidi A, Slightom J, Gonsalves D (1995) Transformation of plum with the papaya ringspot virus coat protein gene and reaction of transgenic plants to plum pox virus. *J Am Soc Hortic Sci* 120:943–952
- Scorza R, Melnicenco L, Dang P, Abbott AG (2002) Testing a microsatellite marker for selection of columnar growth habit in peach [*Prunus persica* (L.) Batsch]. *Acta Hort* 592:285–289
- Smigocki AC, Hammerschlag FA (1991) Regeneration of plants from peach embryo cells infected with a shooty mutant strain of *Agrobacterium*. *J Am Soc Hortic Sci* 116:1092–1097
- Snir I (1982) In vitro propagation of sweet cherry cultivars. *HortScience* 17:192–193
- Snir I (1984) In vitro propagation of ‘Canino’ apricot. *HortScience* 19:229–230
- Song GQ, Sink KC (2006) Transformation of Montmorency sour cherry (*Prunus cerasus* L.) and Gisela 6 (*P. cerasus* × *P. canescens*) cherry rootstock mediated by *Agrobacterium tumefaciens*. *Plant Cell Rep* 25:117–123
- Soriano JM, Vilanova S, Romero C, Llácer G, Badenes ML (2005) Characterization and mapping of NBS-LRR resistance gene analogs in apricot (*Prunus armeniaca* L.). *Theor Appl Genet* 110:980–989
- Sosinski B, Gannavarapu M, Hager LD, Beck LE, King GJ, Ryder CD, Rajapakse S, Baird WV, Ballard RE, Abbott AG (2000) Characterization of microsatellite markers in peach [*Prunus persica* (L.) Batsch]. *Theor Appl Genet* 101:421–428
- Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318:624–629
- Tabachnik L, Kester DE (1977) Shoot culture for almond and almond-peach hybrid clones in vitro. *HortScience* 12:545–547
- Tang HR, Ren ZL, Reustle G, Krczal G (2002) Plant regeneration from leaves of sweet and sour cherry cultivars. *Sci Hort* 93:235–244
- Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res* 17:6463–6471
- Testolin R, Marrazzo T, Cipriani G, Quarta R, Verde I, Dettori MT, Pancaldi M, Sansavini S (2000) Microsatellite DNA in peach (*Prunus persica* L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. *Genome* 43:512–520
- Valentine L (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. *Plant Physiol* 133:948–955
- Vilanova S, Romero C, Abernathy D, Abbott AG, Burgos L, Llácer G, Badenes ML (2003a) Construction and application of a bacterial artificial chromosome (BAC) library of *Prunus armeniaca* L. for the identification of clones linked to the self-incompatibility locus. *Mol Gen Genomics* 269:685–691
- Vilanova S, Romero C, Abbott AG, Llácer G, Badenes ML (2003b) An apricot (*Prunus armeniaca* L.) F<sub>2</sub> progeny linkage map based on SSR and AFLP markers, mapping plum pox virus resistance and self-incompatibility traits. *Theor Appl Genet* 107:239–247
- Viruel MA, Messeguer R, de Vicente MC, García-Más J, Puigdomènech P, Vargas FJ, Arús P (1995) A linkage map with RFLP and isozyme markers for almond. *Theor Appl Genet* 1:964–971

- Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Wang Q, Zhang K, Qu X, Jia J, Shi J, Jin D (2001) Construction and characterization of a bacterial artificial chromosome library of peach. *Theor Appl Genet* 103:1174–1179
- Webster AD, Wertheim SJ (1993) Comparisons of species and hybrid rootstocks for European plum cultivars. *J Hortic Sci* 68:861–869
- Williams JGK, Kubelik AR, Livak JK, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531–6535
- Yamamoto T, Shimada T, Imai T, Yaegaki H, Haji T, Matsuta N, Yamaguchi M, Hayashi T (2001) Characterization of morphological traits based on a genetic linkage map in peach. *Breed Sci* 51:271–278
- Yancheva SD, Druart P, Watillon B (2002) *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.). *Acta Hort* 577:215–217
- Ye XJ, Brown SK, Scorza R, Cordts JM, Sanford JC (1994) Genetic transformation of peach tissues by particle bombardment. *J Am Soc Hortic Sci* 119:367–373
- Zhebentyayeva T, Horn R, Mook J, Lecouls AC, Georgi L, Swire-Clark G, Baird V, Reighard G, Abbott A (2004) Physical mapping of the peach genome: a current status of an integrated physical/genetic map. *Proc 2nd Int Rosaceae Genome Mapping Conf*, Clemson University, May 22–24, Clemson, South Carolina
- Zimmerman RH, Debergh PC (1991) Micropropagation of temperate zone fruit and nut crops. In: Zimmerman RH, Debergh P (eds) *Micropropagation: technology and application*. Kluwer, Boston, pp 231–246
- Zuo J, Niu QW, Ikeda Y, Chua NH (2002) Marker-free transformation: increasing transformation frequency by the use of regeneration-promoting genes. *Curr Opin Biotechnol* 13:173–180

## I.12 Strawberry

J.A. MERCADO, F. PLIEGO-ALFARO, and M.A. QUESADA<sup>1</sup>

### 1 Introduction

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is an octoploid species ( $2n = 8x = 56$ ) belonging to the genus *Fragaria* of the family Rosaceae. This genus comprises at least 15 species (Hancock 1990) from which two wild species, *F. vesca* and *F. moschata*, are also commercially grown on a reduced scale. The modern cultivated strawberry arose in Europe in the 18th century as a chance cross between two American native species, *F. virginiana* and *F. chiloensis*. In spite of its recent origin and the restricted location of its progenitors, breeders have developed new cultivars adapted to a wide variety of environmental conditions. Currently, strawberry is grown in most arable regions of the world.

Although strawberry is not an essential component of the diet, its delicious flavor and taste, attractive appearance and seasonal availability make this fruit an excellent crop. Even more, strawberries are rich in phytochemical compounds with potential antioxidant compounds, mainly ellagic acid and flavonoids, which can lower the risk of cardiovascular events and tumorigenesis (Hannum 2004). These qualities have ensured that the economic importance of this crop has increased throughout the world and, nowadays, it remains as a crop of primary interest for both research and fruit production. However, the production practices conventionally used are not considered to be sustainable. Strawberry cultivation requires previous soil fumigation to be successful, uses large amounts of plastic for controlling weeds and plant growth, has a high water demand and depends on frequent applications of pesticides (Pritts 2002). Furthermore, the fruit is considered by consumers as one of the most inconsistent items in the marketplace. Therefore, lack of resistance to diseases and environmental stresses, fruit quality and postharvest handling are the major challenges in strawberry production. Most breeding programs are devoted to addressing these problems (Faedi et al. 2002), but conventional strawberry breeding is hampered by the octoploid nature and high heterozygosity of this species. Furthermore, commercial strawberry has a relative narrow germplasm base, and most genes in modern cultivars derive from a few nuclear and cytoplasmic sources (Sjulin and Dale 1987; Dale and Sjulin 1990). For these reasons, wild species are being included in breeding

<sup>1</sup> Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga, Spain, e-mail: mercado@uma.es

programs in order to expand the germplasm (Faedi et al. 2002; Hancock et al. 2002; Marta et al. 2004).

In recent years, biotechnological approaches, such as marker assisted selection, have been integrated progressively into breeding programs, and used successfully for cultivar recognition and phylogenetic studies (Faedi et al. 2002; Graham 2005). The use of molecular markers to generate linkage maps has also provided promising results in the selection of superior genotypes, as in the case of resistance to the red stele disease caused by *Phytophthora fragariae*, one pathogen of high concern (Haymes et al. 2000).

Since 1990, when the first reports on genetic transformation of strawberry were published, transformation protocols have been developed for many cultivars. Several genes of interest controlling crucial traits such as fruit shelf life and pest resistance have been introduced into strawberry. Hence, biotechnological improvement of strawberry through genetic transformation appears a powerful tool that can circumvent some of the problems inherent in classical breeding. In addition to extending the germplasm available to improve this species, desired traits can be introduced directly into an elite cultivar without sexual recombination, since strawberry is propagated vegetatively by runners rather than by seed. This chapter outlines the major trends in the genetic transformation of strawberry, with special attention given to those biotechnological advances that could be useful in its genetic improvement.

## 2 Economic Importance

Strawberry world production has shown a significant increase in the last 25 years. From 1980–2004, the land cultivated with strawberry increased by 25% and fruit production by 73%. In 2004, strawberry was cultivated on 214,118 ha, reaching a production of 3.1 million tonnes (FAOSTAT 2005, [http://www.fao.org/waicent/portal/statistics\\_en.asp](http://www.fao.org/waicent/portal/statistics_en.asp)). Fifty seven countries contributed to this production, though only nine of them accounted for more than 72% of the total. During the period 2000–2004, average world production was 3.17 million tonnes, the major strawberry producer nations being the USA (830,000 t year<sup>-1</sup>, 26.2% of the world production and 8.7% of world area), Spain (307,000 t year<sup>-1</sup>, 9.7% and 4.2%), Korea (210,000 t year<sup>-1</sup>, 6.6% and 3.9%), Japan (206,000 t year<sup>-1</sup>, 6.5% and 3.3%), Italy (167,000 t year<sup>-1</sup>, 5.3% and 2.9%), Poland (160,000 t year, 5.2% and 22.6%), Mexico (143,000 t year<sup>-1</sup>, 4.5% and 2.6%), Turkey (136,000 t year<sup>-1</sup>, 4.3% and 5%) and the Russian Federation (135,000 t year<sup>-1</sup>, 4.2% and 5.3%). A comparison of these data with those reported by Faedi et al. (2002) for the period 1995–1999 shows a slight increase in production for most countries, especially Korea and Turkey, with a slight reduction for Italy and Poland. In terms of yield per hectare, the USA and Spain showed the highest yield in the period 2000–2004, with 43 and 33 t ha<sup>-1</sup>, respectively. Turkey, the Russian Federation and, especially Poland, have lower



yields at 12, 11.6 and 3.3 t ha<sup>-1</sup>, respectively. The total value of USA production was 1.22 billion dollars in 2002, being the third fruit in value in the noncitrus category behind grapes and apples (Sjulin 2003). It is noteworthy that most strawberry production is localized in areas with mild winter climates (Faedi et al. 2002).

Approximately 15% of world strawberry production is exported as fresh fruit and, at least in the USA, more than 25% is processed. The major fresh strawberry exporters are Spain (212,300 t in 2003, 81% of its strawberry production) and the USA (94,600 t, 11.2%). Spain is the main supplier for the European market during the winter period (February and March) and Germany, France and the UK are the major consumers of this production. In Spain, as well as other Mediterranean nations, the concentration of strawberry production over short periods often creates a product excess, reducing retail prices (Faedi et al. 2002). In the case of the USA, exported fruits are destined mainly for Canada and a small quantity for Japan and Mexico (USDA 2004). California has more than 85% of the USA strawberry production (Sjulin 2003). This region also supports the most active and successful strawberry breeding programs. In fact, the program of the University of California has released very popular varieties (e.g. Camarosa, Chandler, Seascape, Selva, Pajaro, Oso Grande), accounting for more than 50% of the world's strawberry production (Faedi et al. 2002).

### 3 Current Research and Development

#### 3.1 In Vitro Tissue Culture

In general, strawberry can be managed easily under in vitro conditions. Meristem tips, generally obtained from runners of virus-free plants, are commonly used to establish in vitro cultures, which are employed for mass propagation or as a source of plant material for regeneration and transformation experiments. Micropropagation of strawberry by axillary buds has been studied intensively for a long time (Boxus 1992). On a commercial scale, tissue culture-derived strawberry plants are estimated to cost four to five times more than plants produced by conventional propagation (George 1996). However, micropropagated strawberry has several advantages, such as its ability to multiply virus-free stock rapidly and, in particular, the improved capacity of these plants to produce runners for planting in the field (López-Aranda et al. 1994; George 1996). By contrast, when used directly in the field for fruit production, micropropagated material shows a decrease in fruit size and weight (López-Aranda et al. 1994). Several parameters used during the in vitro phase can affect the behavior of micropropagated strawberry in the nursery, e.g. plant genotype, mineral formulation, type and concentration of cytokinin in the medium and the number of subcultures. It is generally recommended not to exceed four to five subcul-



tures to avoid loss of trueness-to-type of the propagated material (Faedi et al. 2002). Zhou et al. (2005) found that rooting strawberry plantlets under photoautotrophy conditions significantly increased plant height and weight after acclimatization. This effect was related to a better photosynthetic performance of *in vitro* plants. Interestingly, rooting of micro shoots can be achieved under *ex vitro* conditions, improving the number and length of runners produced (Borkowska 2001).

Adventitious shoot regeneration has been achieved in several cultivars using a broad range of explants. These include leaf explants (Jones et al. 1988; Nehra et al. 1989; Sorvari et al. 1993; Barceló et al. 1998), petioles (Jones et al. 1988; James et al. 1990; Rugini and Orlando 1992), stipules (Rugini and Orlando 1992), stem tissue (Graham et al. 1995; Mathews et al. 1995), runners (Liu and Sanford 1988), mesophyll protoplasts (Nyman and Wallin 1988), anthers (Owen and Miller 1996), cotyledons (Miller and Chandler 1990), roots (Rugini and Orlando 1992) and immature embryos (Wang et al. 1984). Passey et al. (2003), testing the regeneration ability of seven commercial cultivars using a range of explants, showed that leaf disks gave the highest regeneration rates, although two genotypes showed a limited ability to regenerate shoots in all explants tested. Rugini and Orlando (1992) also found great differences in shoot regeneration ability from calluses among cultivars when leaf, petiole and root tissues were used as initial explants. However, these differences disappeared when whole leaves, including stipules, were used as explants. The variation in the regeneration capacity amongst different cultivars has also been observed in other studies (Jones et al. 1988; Nehra et al. 1989, 1990a), indicating that a strong genetic component determines the success of adventitious regeneration. Recently, a role has been suggested for endogenous  $H_2O_2$  in the process of bud primordia formation from strawberry calli (Tian et al. 2003). Along this line, Yonghua et al. (2005) found that shoot regeneration was enhanced when explants were cultured under red or green plastic films, and this was correlated with an increase in activity of antioxidant enzymes and endogenous hormone concentration.

Besides micropropagation and shoot regeneration, somaclonal variation induced by *in vitro* culture has been used by several authors to accelerate the breeding process. In this regard, resistances of *in vitro*-derived plants to fungal pathogens (Toyoda et al. 1991; Sowik et al. 2001; Hammerschlag et al. 2006), low temperature (Rugienius and Stanys 2001) and salt stress (Dziadczyk et al. 2003) have been studied with promising results.

### 3.2 Transgenic Technology

The feasibility of *in vitro* strawberry culture has allowed the optimization of transformation protocols for a large number of commercial cultivars, including some of the most popular genotypes such as Chandler, Camarosa, Elsanta and Pajaro. Genetic transformation of cultivated strawberry was first reported

in 1990 by two independent groups (James et al. 1990; Nehra et al. 1990b), both using an *Agrobacterium*-mediated system. Nowadays, this is the transformation method most often employed, although other strategies have been described. It is noteworthy that a method for *Agrobacterium* transformation of strawberry using culture media containing glucose or fructose is protected under US patent number 6,274,791. Besides commercial strawberry, successful transformation has been reported in *F. vesca* (El Mansouri et al. 1996; Haymes and Davis 1998; Alsheikh et al. 2002) and *F. moschata* (Mezzetti et al. 2002). In general, genetic transformation of strawberry is highly genotype dependent.

### 3.2.1 *Agrobacterium*-Mediated Transformation

#### 3.2.1.1 Explant

Several strawberry cultivars have been transformed following *A. tumefaciens* infection, with variable efficiencies ranging from 0.1% in the cv. Rapella (James et al. 1990) to 58% in the cv. Totem (Mathews et al. 1995). Most transformation experiments, in both cultivated and wild strawberry, have been performed using leaf disks as explants (James et al. 1990; Nehra et al. 1990b; El Mansouri et al. 1996; du Plessis et al. 1997; Martinelli et al. 1997; Barceló et al. 1998; Puite and Schaart 1998; Wawrzynczak et al. 2000; Alsheikh et al. 2002; Ricardo et al. 2003; Gruchala et al. 2004). Barceló et al. (1998) reported that leaf disks from seedlings of the cv. Chandler were transformed more easily than leaves from shoots micropropagated in the presence of benzyladenine (BA), probably due to the higher regeneration capacity of juvenile material. However, when BA was replaced by kinetin the transformation rate was similar to that obtained with juvenile leaves. In the cv. Rapella, petioles were transformed more efficiently than leaf explants, in spite of their lower regeneration rate (James et al. 1990). In contrast, leaf disks of *F. vesca* gave higher transformation rates than petioles (Alsheikh et al. 2002). Stipules of micropropagated shoots have also been transformed by *Agrobacterium* infection (Monticelli et al. 2002; Chalavi et al. 2003). Mathews et al. (1995, 1998) used meristematic sections at the base of in vitro proliferating plantlets of the cvs. Tristar and Totem. These explants contained several buds and young shoot segments, and the transformation rates obtained, ranging from 13.6–58.8%, were significantly more than those obtained with leaf explants. However, a high percentage of the primary transformants obtained through continuous selection under 25 mg l<sup>-1</sup> kanamycin were chimeras, containing transgenic and non-transgenic sections. Graham et al. (1995) also used stem tissue from the base of in vitro-derived plants of different cultivars, although in this case the regeneration rate in 10 mg l<sup>-1</sup> kanamycin was low.

### 3.2.1.2 Preculture and *Agrobacterium* Virulence

Preculture of explants in adequate conditions prior to *Agrobacterium* infection can improve transformation by increasing the number of cells competent for regeneration and transgene integration (Birch 1997). In the cv. Chandler, a preculture period of 10 days in the regeneration medium was optimal for recovering kanamycin resistant shoots (Barceló et al. 1998). Asao et al. (1997) cultured leaf and petiole explants for 1 day in liquid medium prior to *A. tumefaciens* inoculation. In *F. vesca*, a 2-day preculture of explants in the regeneration medium was employed (El Mansouri et al. 1996; Alsheikh et al. 2002). The effect of increasing *Agrobacterium* virulence on strawberry transformation has also been studied. Alsheikh et al. (2002) found a significant improvement in the frequency of GUS positive plants in *F. vesca semperflorens* through the inclusion of acetosyringone (AS) during the culture of *A. tumefaciens*. Gruchala et al. (2004) also found a slight increase in the transformation rate in explants of *Fragaria* × *ananassa* infected with bacteria precultured with AS or indole acetic acid (IAA). In contrast, James et al. (1990) did not detect any effect of AS on the percentage of explants that produced kanamycin-resistant callus after 100 days of culture. Unfortunately, the efficiency of different *A. tumefaciens* strains on strawberry transformation has received little attention, and no comparative study of different strains has been reported.

### 3.2.1.3 Selection Strategies

Most binary vectors used to transform strawberry are derived from pBIN19 (Bevan 1984) and contain the *nptII* gene for kanamycin selection of transgenic shoots. In general, strawberry tissues are extremely sensitive to kanamycin. Shoot regeneration from leaf disks is impaired at kanamycin concentrations as low as 10 mg l<sup>-1</sup> (El Mansouri et al. 1996; Barceló et al. 1998; Gruchala et al. 2004), but a concentration of 25–50 mg l<sup>-1</sup> is generally employed to avoid the risk of recovering non-transgenic shoots. In most protocols, a period of 2–10 days without kanamycin selection after *Agrobacterium* infection is included to allow single transformed cells to express the transgenes and to form cell clusters that better withstand the selection pressure. As previously mentioned, Mathews et al. (1995, 1998) found a high percentage of chimaerism in regenerated shoots of the cv. Totem, using continuous selection on 25 mg l<sup>-1</sup> kanamycin. This was probably due to the high antibiotic tolerance of this particular cultivar, since non-transformed shoots were able to grow and proliferate at this kanamycin concentration. In order to recover pure transformants, these authors developed a regeneration procedure in which leaf explants from primary transgenics were subjected to three regeneration cycles, increasing the kanamycin concentration from 40 mg l<sup>-1</sup> in the first round of regeneration to 80 mg l<sup>-1</sup> in the third round (Mathews et al. 1998). Regenerated shoots after this regeneration process were able to grow and root at high kanamycin concentration (120 mg l<sup>-1</sup>). This transformation protocol is protected under US

patent no. 5,750,870. Monticelli et al. (2002) used a similar strategy to obtain uniform transgenic plants with only two rounds of regeneration. A high proportion of chimaerism has also been obtained by Chalavi et al. (2003) when using stipules as explants, although chimeric shoots bleached after three to four subcultures in the presence of kanamycin. Contrary to these results, the presence of chimerism in transgenic strawberry regenerated from leaf disks has not been described elsewhere and, therefore, it seems that a continuous selection pressure, adjusting kanamycin concentration to the sensitivity of the explants, is enough to ensure the recovering of pure transgenics.

#### 3.2.1.4 Elimination of Marker Genes

Regulatory authorities and consumers are demanding that transgenic plants to be commercialized are devoid of unnecessary genes, e.g. selectable marker genes or vector backbone sequences. In the case of vegetatively propagated plants, such as strawberry, the elimination of marker genes can be performed by transposition or by site-specific recombination. Recently, Schaart et al. (2004) reported the production of marker-free transgenic strawberry plants using an inducible site-specific recombination system. They constructed a binary vector containing a plant-adapted recombinase (R) gene and a bifunctional selectable marker created by the combination of the *nptII* gene for kanamycin selection and *codA* gene that confers sensitivity to 5-fluorocytosine (5-FC). Both genes were located adjacent and flanked by directly repeated recombination sites (Rs). The Rs-flanked fragment was inserted between an enhanced 35S promoter and *gus* gene. The expression of the recombinase gene was induced by application of dexamethasone (DEX), leading to the excision of the Rs-flanked fragment (Schaart et al. 2004). These authors assessed two strategies for recovering marker-free plants. In the first one, leaf explants were treated with DEX to induce recombinase activity 1 month after kanamycin selection and, subsequently, explants were cultured in the presence of 5-FC to eliminate cells that still retained the selection marker. In the alternative strategy, shoots were regenerated in the presence of kanamycin, and leaf explants from these transgenic shoots were treated with DEX and subsequently subjected to a second round of regeneration in medium supplemented with 5-FC (Schaart et al. 2004). This selection strategy proved to be more efficient than the early selection, and more than 30 putative marker-free plants were recovered. Molecular analysis of several of these lines confirmed that they did not contain marker genes.

#### 3.2.2 Direct Gene Transformation

As strawberry is easily transformed via *Agrobacterium* infection, methods of direct gene transformation have received little attention in this species. Nyman and Wallin (1992) reported the transformation of leaf and petiole protoplasts by electroporation, obtaining a transformation frequency of  $1-5 \times 10^{-4}$  se-

lected calli per plated protoplast. Biolistic bombardment was used by Wang et al. (2004) to transform strawberry calli derived from anther culture. In this study, after 40 days of selection in  $10 \text{ mg l}^{-1}$  phosphinotricin, an average of 5.4 resistant calli per bombarded plate was recorded. In the case of leaf explants, Cordero de Mesa et al. (2000) found transient GUS expression 5 days after biolistic bombardment, although none formed callus in the presence of kanamycin. The latter authors developed a novel approach to obtain higher transformation rates. They subjected leaf explants to biolistic bombardment using microprojectiles coated with *A. tumefaciens* cells instead of naked plasmid. The transformation rate increased from 4% when using standard *Agrobacterium* infection to 15% by using this *Agrobacterium*-biolistic combined method.

### 3.3 Molecular Genetics

#### 3.3.1 Fruit Ripening

In recent years, several research groups have focused on strawberry molecular genetics, and a number of genes have been isolated and cloned. Most of these investigations have been devoted to studying fruit ripening, not only owing to the high commercial interest of this process, but also because strawberry is considered to be a model for physiological studies of non-climacteric fruit development (Manning 1994; Perkins-Veazie 1995). Strawberry fruit develops from the floral receptacle, with the true fruit, the so-called achene, growing on the outer surface. Achenes regulate the growth of the receptacle through the synthesis of auxin, and ripening is associated with a gradual depletion of the supply of auxin in the latter stages of growth (Perkins-Veazie 1995). Thus, most ripening-related genes are inhibited by auxin treatment (Manning 1994, 1998). Manning (1998) used cDNA from ripe and white strawberry fruits to screen a cDNA library prepared from ripe fruit. He isolated 66 ripening-related clones, belonging to 26 families of non-redundant genes. These genes encoded proteins involved in key metabolic processes including anthocyanin synthesis, cell wall degradation, sucrose and lipid metabolism, synthesis and degradation of proteins and respiration. A number of these genes were novel to fruits. Nam et al. (1999) used *F. vesca* as a model system to study strawberry ripening. They constructed a cDNA library from deached ripe receptacles, which was screened differentially with cDNAs from either ripe or unripe fruits. They identified 192 clones scored as ripening-enhanced or -repressed transcripts, nine of which were analyzed in detail. Apparently, none of these genes was directly related to major ripening processes and, rather, their sequences indicated a wide range of putative functions, e.g. lipid and amino acid biosynthesis, secondary metabolism or host defense response. Several of these genes were also expressed in commercial strawberry fruits, although their expression patterns were slightly different.

DNA microarray technology provides a powerful means to study in a single experiment the expression profile of thousands of genes. Aharoni et al. (2000) constructed strawberry microarrays using 1701 cDNAs, comprising 1100 sequenced ESTs (expressed sequence tags) and 601 unsequenced cDNAs, obtained from ripe fruit including achenes. These microarrays have been used to monitor gene expression in strawberry fruit under different experimental conditions, comparing developmental stages (Aharoni et al. 2000), receptacle versus achene tissues (Aharoni and O'Connell 2002), auxin treated and non-treated fruits (Aharoni et al. 2002) and cultivars differing in fruit firmness (Salentijn et al. 2003). An alcohol acyltransferase (SAAT) gene, involved in strawberry fruit flavor, was identified using this technology (Aharoni et al. 2000). This gene was introduced in petunia to assess its potential for metabolic engineering of volatile ester production (Beekwilder et al. 2004). Other genes that could be involved in aroma biogenesis have also been identified (Iannetta et al. 2004; Moyano et al. 2004).

One of the most important events in strawberry ripening is fruit softening, since the rapid loss of firm texture that occurs in this fruit limits its postharvest shelf life. It is generally believed that fruit softening occurs mainly by the action of cell wall modifying enzymes. Several genes involved in this process have been isolated. Glucanases, enzymes that modify the hemicellulose network, have been studied most extensively. At least two endo- $\beta$ -1,4-glucanases are up-regulated during strawberry ripening (Harpster et al. 1998; Llop-Tous et al. 1999; Trainotti et al. 1999; Woolley et al. 2001). One of them, *FaEG1*, is specific to the ripening process, while the other gene, *FaEG3*, is also expressed in green fruit and young vegetative tissue (Trainotti et al. 1999). A fruit-specific expansin gene was cloned by Civello et al. (1999). It is thought that these proteins disrupt hydrogen bonds between cellulose and hemicellulose microfibrilles, regulating the accessibility of other enzymes to their substrate. The strawberry expansin was not down-regulated by auxin and apparently its expression was not a determinant of fruit softening. Genes related to pectin metabolism have also been studied. Redondo-Nevado et al. (2001) reported the isolation of a fruit-specific endopolygalacturonase (PG) gene that was expressed at the onset of fruit ripening. Its expression pattern suggests a role in the release of oligosaccharins, biologically active molecules involved in fruit ripening, rather than pectin depolymerization. Similar to PG, pectate lyase also catalyzes the cleavage of de-esterified pectins. Three ripening-related pectate lyase genes have been identified in strawberry (Medina-Escobar et al. 1997; Benítez-Burraco et al. 2003), and their expression was strongly reduced in ripe fruits stored at high concentrations of carbon dioxide, a postharvest treatment that extends shelf life and increases fruit firmness. Pectin esterase catalyzes the demethylation of pectins, a prerequisite to the action of PG or pectate lyase. Castillejo et al. (2004) isolated four pectin esterase cDNAs, *FaPE1* to *FaPE4*, which showed different expression patterns. Only one of them, *FaPE1*, was detected exclusively in ripening fruit. A frequent observation during ripening of fleshy fruits is the loss of galactose found in side chains of pectins. In



strawberry, Trainotti et al. (2001) reported the isolation of three different  $\beta$ -galactosidases. All were detected in fruits and vegetative tissues, although only one of them, *Fa $\beta$ gal1*, showed increased expression during the ripening stages. A ripening-related  $\beta$ -xylosidase gene has been also cloned, but the cell wall target of this enzyme was unclear (Martínez et al. 2004). Other genes related to the ripening process have been isolated, including a dihydroflavonol 4-reductase (Moyano et al. 1998) involved in anthocyanin synthesis, a D-galacturonate reductase whose expression is correlated with ascorbic acid content (Agius et al. 2003), an HyPRP protein (Blanco-Portales et al. 2004), a low molecular weight heat shock protein (Medina-Escobar et al. 1998), an auxin-binding protein (Lazarus and Macdonald 1996) and two genes that also respond to environmental stress. The latter are a calcium-dependent protein kinase (Llop-Tous et al. 2002) and a lipid transfer protein (Yubero-Serrano et al. 2003).

### 3.3.2 Plant Stress

Besides fruit ripening, other physiological processes have been studied at the gene level. Genes differentially expressed in cold acclimated plants were isolated by Ndong et al. (1997). Recently, two genes have been identified that are involved in strawberry defense response. Martínez et al. (2005) isolated a phyto-cystatin gene expressed in mature leaves, roots and achenes, that showed antifungal activity in vitro against *Botrytis cinerea* and *Fusarium oxysporum*. Mehli et al. (2004) reported the isolation of a gene encoding a polygalacturonase-inhibiting protein. This gene was highly expressed in mature fruit and induced after inoculation with *B. cinerea*.

### 3.3.3 Promoters

Functional analyses of homologous promoters in strawberry are scarce. Spolaore et al. (2003) studied promoters of the endoglucanase genes, *FaEG1* and *FaEG3*, using a transient expression method consisting of injecting fruit with a suspension of *Agrobacterium* cells containing plasmids to be analyzed. Agius et al. (2005) performed transient expression assays to study the activity of D-galacturonate reductase promoter using biolistic. Transgenic strawberry plants have also been generated to evaluate the usefulness of heterologous promoters. Along these lines, Schaart et al. (2002) tested a receptacle promoter from petunia (*FBP7*) to assess its utility as a fruit-specific promoter in strawberry. Transgenic plants showed GUS activity in reproductive but not in vegetative tissues, although in red fruit the 35S promoter was six-fold stronger than the *FBP7* promoter. Cordero de Mesa et al. (2004) analyzed the expression of the 35S promoter in flowers and pollen of transgenic plants. They found an increased GUS activity in mature pollen and in grains stored for 5 weeks at 25 °C. Finally, in order to achieve tolerance to diseases caused by phytoplasmas,



prokaryotes restricted to sieve elements, Zhao et al. (2004) tested a phloem-specific promoter from *Arabidopsis*. In this case, GUS activity was restricted to phloem tissues of transgenic plants.

## 4 Practical Applications of Transgenic Plants

Since 1994, at least 48 field trials with transgenic strawberry plants have been performed, 42 in USA and 6 in Europe. Most of these assays have been devoted to improving fungal resistance (40% of total field trials), herbicide tolerance (20%) and fruit quality (18%). This distribution accurately reflects the main concerns of the strawberry industry, since these targets, pest and disease resistance as well as fruit quality and yield, are general objectives of most breeding programs (Watt et al. 1999; Faedi et al. 2002).

### 4.1 Biotic Stress

Disease and pest resistance are two of the main problems of strawberry cultivation. This crop needs almost complete soil sterilization to assure good fruit yield, and fumigation is achieved generally with methyl bromide. However, due to its negative effects on the environment, its use has been forbidden in most developed countries under the Montreal Protocol. A complete loss of soil fumigation would have a major impact on the strawberry industry, with an estimated 35–50% yield loss (Sjulin 2003). A tremendous effort is being made to seek more environmentally friendly procedures, including the introduction of resistant varieties. A summary of the main strawberry pests and status of resistance in cultivated and wild species is given in Roskopf (1999). Transgenic strawberry plants with disease-resistant genes could help to address this problem in the long term. Additionally, this technology would reduce the use of pesticides, enhancing the safety and quality of this food crop.

#### 4.1.1 Fungal Resistance

Amongst the different strawberry pathogens, fungi are the most important in terms of yield loss. Major fungal diseases include red stele (*Phytophthora fragariae*), *Verticillium* wilt, leaf spot (*Mycosphaerella fragariae*), leaf scorch (*Diplocarpon earlianum*), powdery mildew (*Sphaerotheca macularis* f. sp. *fragariae*), gray mold (*Botrytis cinerea*) and anthracnose crown rot (*Colletotrichum* spp.) (Maas 1998; Roskopf 1999). Few of these diseases have been targeted by genetic engineering. Chalavi et al. (2003) introduced a chitinase gene from *Lycopersicon chilense* into the cv. Joilette, to enhance resistance to *Verticillium dahliae*. The three transgenic lines obtained showed high levels of chitinase mRNA expression, and a significant low rate of crown infection.

Visual wilting symptoms were slightly lower in two transgenic lines when compared to the control. Asao et al. (1997) obtained transgenic strawberry with enhanced resistance to *Sphaerotheca humuli* through the expression of a rice chitinase gene. Protein extraction of transgenic leaves showed a three-fold increase in chitinase activity in comparison to control plants. After inoculation with fungal spores, the percentage of leaf surface with disease symptoms was reduced from 40% in the control to 22% in transgenic plants. Apparently, there was a correlation between chitinase activity and leaf area with lesions. Morphological characteristics, as well as yield of transgenic plants, were similar to the control and, additionally, no significant environmental effect on the growth of other plants and microflora was detected (Asao et al. 2003). Thaumatin II from *Thaumatococcus danielli*, a pathogenesis-related protein showing antifungal activity, also associated with sweet taste, has been introduced into strawberry cv. Firework (Shestibratov and Dolgov 2005). Transgenic plants showed enhanced resistance against *Botrytis cinerea* assayed with a leaf disk test. Recently, Vellicce et al. (2006) described the obtainment of transgenic plants overexpressing a chitinase gene from *Phaseolus vulgaris*. Transgenic plants showed an enhanced resistance to *Botrytis* but not towards *Colletotrichum acutatum*. Plants of the cv. Camarosa have been transformed with a chitinase gene from the soil fungus *Trichoderma* to obtain *Colletotrichum* tolerance (Yubero-Serrano et al. 2002). Several transgenic lines showed a significant reduction in *Colletotrichum* crown rot when compared to control plants.

#### 4.1.2 Insect and Virus Resistance

Insect resistance in strawberry has been accomplished by insertion of antifeedant genes. James et al. (1992) and Graham et al. (1995) obtained transgenic plants expressing a Cowpea protease inhibitor gene (*CpTi*). This gene is effective against a wide range of Lepidopteran and Coleopteran insects. As in the case of other protease inhibitors, it forms complexes with proteinases, inhibiting proteolytic activity and therefore interfering with normal larval development (Watt et al. 1999). Transgenic strawberries expressing *CpTi* have been assayed for resistance to damage by vine weevil larvae (*Otiorynchus* spp.) with contrasting results. This is one of the major pests in strawberry for which resistance is not available in either cultivated or wild species (Roskopf 1999). In the report of James et al. (1992), analysis of six transgenic lines showed no reduction in the survival rate of vine weevil larvae. By contrast, Graham et al. (1997, 2002) found that transgenic plants were less damaged than controls when infected by weevil larvae under glasshouse and field conditions, with no significant impact on non-target pests. A lectin gene from *Galanthus nivalis*, a different anti-insect protein, has also been introduced in strawberry, alone or in combination with *CpTi*, although transgenic plants failed to show resistance to weevil attack (Graham 2005).

In the case of virus, Finstad and Martin (1995) described the transformation of strawberry with the coat protein gene from strawberry mild yellow edge virus, although no assessment of virus tolerance was reported.

#### 4.1.3 Herbicide Tolerance

Transgenic strawberry plants tolerant to glyphosate, the active compound of the herbicide Roundup, have been obtained by Morgan et al. (2002). Glyphosate inhibits EPSP (enolpyruvyl shikimate phosphate synthase), a key enzyme in the aromatic amino acid pathway. These authors transformed leaf explants with an EPSP synthase gene found in the C24 strain of *Agrobacterium tumefaciens* which is tolerant to glyphosate. Transgenic plants showed a wide variation in tolerance to Roundup spray, and 30 highly tolerant lines were selected for field trials. Du Plessis et al. (1997) obtained transgenic plants resistant to glufosinate through the expression of the phosphinotricin acetyl transferase gene. Four out of 22 herbicide-resistant lines resembled the control phenotype in field trials under commercial conditions.

#### 4.2 Abiotic Stress

Different late embryogenesis-abundant (LEA) proteins have been introduced into strawberry to increase salt and freezing tolerances. Wang et al. (2004) produced transgenic plants expressing a LEA3 protein from barley. Salt tolerance tests were conducted under in vitro conditions. They observed that wilting after 7 days of culture in medium supplemented with 50 and 100 mM NaCl was significantly lower in transgenic plants. Survival rate after salt exposure was also higher in LEA3 expressing lines. Over-expression of the acidic dehydrin *wcor410* obtained from wheat, a different class of LEA protein, improved freezing tolerance of cold acclimated strawberry leaves (Houde et al. 2004). It has been proposed that this protein prevents the destabilization of plasma membrane that occurs during the dehydration associated with freezing. In the three transgenic lines obtained that expressed the protein at high level, the threshold temperature of freezing damage was lowered 5 °C, from -12 to -17 °C. However, this higher tolerance was not observed in leaves not cold acclimated, suggesting that other factors could be necessary to activate the WCOR410 protein. Similarly, when whole plants were subjected to freezing conditions, transgenic plants collapsed during the recovery phase at 20 °C, although the leaves were healthy, indicating that WCOR410 protein did not exert any beneficial effect on roots. Owens et al. (2002) obtained two transgenic lines over-expressing a cold-induced transcription factor, *CBF1*. Freezing tolerance of leaf disks was significantly improved in both lines, but this effect was not observed in receptacles.

### 4.3 Fruit Quality and Yield

Several biotechnological attempts have been made to control ripening and delay fruit softening. Ethylene production does not appear to be associated with strawberry ripening and, thus, this fruit has been classified as non-climacteric (Perkins-Veazie 1995). However, some evidence indicates that ethylene may play a role in strawberry ripening, and, therefore, the reduction of ethylene biosynthesis could increase postharvest shelf life. Mathews et al. (1995) transformed strawberry with the S-adenosylmethionine hydrolase from T3 bacteriophage. This gene limits the biosynthesis of ethylene by reducing the level of its precursors. Although the presence of transgenes was confirmed in transgenic plants, Mathews and colleagues did not report an effect of gene expression on fruit shelf life. An alternative approach to control fruit softening is the inhibition of proteins involved in fruit cell wall degradation. Jiménez-Bermúdez et al. (2002) obtained transgenic plants of the cv. Chandler, containing an antisense sequence of a pectate lyase gene. Most transgenic lines produced fruits significantly firmer than the control at the stage of full ripeness. Moreover, postharvest softening was diminished in transgenic fruit, extending their shelf life. Additionally, the quality of strawberry jam prepared with these transgenic fruits was improved as a result of a lower disintegration of fruit tissue during jam cooking (Sesmero et al. 2007). Contrary to these results, the inhibition of cellulase genes does not modify substantially the softening behavior of strawberry. Woolley et al. (2001) inhibited the expression of the cellulase gene *Cel1* by both antisense and sense transformation. This gene is expressed specifically in ripening fruits, but its inhibition reduced neither cellulase activity nor fruit softening. Antisense inhibition of *Cel2* did not modify fruit firmness (Pliego-Alfaro et al. 2002).

In order to increase fruit yield, Mezzetti et al. (2004) transformed *F. × ananassa* and *F. vesca* with the *DefH9-iaaM* chimeric gene. This gene is composed of the regulatory region of the *DefH9* gene from snapdragon, which promotes expression in placenta/ovules, and the coding region of the *iaaM* from *Pseudomonas syringae*, an auxin-producing gene. Transgenic plants showed an increased number of inflorescences and also more flowers per inflorescence. Additionally, fruit size and weight was increased. Overall, fruit yield was increased by 180% in cultivated strawberry and by 140% in wild strawberry. Wawrzynczak et al. (2005) also modified auxin metabolism in strawberry through transformation with maize IAA-glucose synthase gene. Transgenic plants showed a dwarf phenotype and this was partially correlated with a reduced level of free IAA.

## 5 Conclusions and Future Challenges

Strawberry genetics has progressed considerably in recent years, in spite of the recalcitrant nature of this plant for molecular analysis. This fact, in combination with the establishment of efficient protocols for genetic transformation of commercial cultivars, has allowed the improvement of several single controlled traits. Marker assisted selection and somaclonal variation are also valuable techniques for strawberry improvement. Resistance to fungal pathogens is nowadays the major challenge of the strawberry industry. Few advances have been made in the search for fungal-resistant genes in strawberry, but recent work with transgenic plants indicates that biotechnology could provide a means to solve this problem in the future. Unfortunately, the development of transgenic strawberries is limited by the poor public acceptance of genetically modified foods, especially fresh products. To overcome this concern, strawberry research should be focused on traits that clearly enhance the safety or nutritional value of this crop. Additionally, searching for genes for selected traits should be extended to related species, to avoid the use of foreign genes in future transformation experiments. Studies on the stability of transgenes in the field and risk assessments are also critical areas for future research.

## References

- Agius F, González-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V (2003) Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nature Biotechnol* 21:177–181
- Agius F, Amaya I, Botella MA, Valpuesta V (2005) Functional analysis of homologous and heterologous promoters in strawberry fruits using transient expression. *J Exp Bot* 56:37–46
- Aharoni A, O'Connell AP (2002) Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. *J Exp Bot* 53:2073–2087
- Aharoni A, Keizer LCP, Bouwmeester HJ, Sun Z, Alvarez-Huerta M, Verhoeven HA, Blaas J, van Houwelingen AMML, De Vos RCH, van der Voet H, Jansen RC, Guis M, Mol J, Davis RW, Schena M, van Tunen AJ, O'Connell AP (2000) Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *Plant Cell* 12:647–661
- Aharoni A, Keizer LCP, Van den Broeck HC, Blanco-Portales R, Muñoz-Blanco J, Bois G, Smit P, De Vos RCH, O'Connell AP (2002) Novel insight into vascular, stress, and auxin-dependent and -independent gene expression programs in strawberry, a non-climacteric fruit. *Plant Physiol* 129:1019–1031
- Alsheikh MK, Suso H-P, Robson M, Battey NH, Wetten A (2002) Appropriate choice of antibiotic and *Agrobacterium* strain improves transformation of antibiotic-sensitive *Fragaria vesca* and *F.v. semperflorens*. *Plant Cell Rep* 20:1173–1180
- Asao H, Nishizawa Y, Arai S, Sato T, Hirai M, Yoshida K, Shinmyo A, Hibi T (1997) Enhanced resistance against a fungal pathogen *Sphaerotheca humuli* in transgenic strawberry expressing a rice chitinase gene. *Plant Biotechnol* 14:145–149
- Asao H, Arai S, Nishizawa Y (2003) Environmental risk evaluation of transgenic strawberry expressing a rice chitinase gene. *Seibutsu-Kogaku Kaishi* 81:57–63

- Barceló M, El Mansouri I, Mercado JA, Quesada MA, Pliego-Alfaro F (1998) Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. *Plant Cell Tissue Organ Cult* 54:29–36
- Beekwilder J, Alvarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester HJ, Aharoni A (2004) Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiol* 135:1865–1878
- Benítez-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero JL, Muñoz-Blanco J (2003) Cloning and characterization of two ripening-related strawberry (*Fragaria* × *ananassa* cv. Chandler) pectate lyase genes. *J Exp Bot* 54:633–645
- Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* 12:8711–8721
- Birch RG (1997) Plant transformation: problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol* 48:297–326
- Blanco-Portales R, López-Ráez JA, Bellido ML, Moyano E, Dorado G, González-Reyes JA, Caballero JL, Muñoz-Blanco J (2004) A strawberry fruit-specific and ripening-related gene codes for a HyPRP protein involved in polyphenol anchoring. *Plant Mol Biol* 55:763–780
- Borkowska B (2001) Morphological and physiological characteristics of micropropagated strawberry plants rooted in vitro or ex vitro. *Sci Hort* 89:195–206
- Boxus P (1992) Mass propagation of strawberry and new alternatives for some horticultural crops. In: Kurata K, Kozai T (eds) *Transplant production systems*. Kluwer, Dordrecht, pp 151–162
- Castillejo C, de la Fuente JI, Iannetta P, Botella MA, Valpuesta V (2004) Pectin esterase gene family in strawberry fruit: study of *FaPE1*, a ripening-specific isoform. *J Exp Bot* 55:909–918
- Chalavi V, Tabaeizadeh Z, Thibodeau P (2003) Enhanced resistance to *Verticillium dahliae* in transgenic strawberry plants expressing a *Lycopersicon chilense* chitinase gene. *J Am Soc Hortic Sci* 128:747–753
- Civello PM, Powell ALT, Sabehat A, Bennett AB (1999) An expansin gene expressed in ripening strawberry fruit. *Plant Physiol* 121:1273–1279
- Cordero de Mesa M, Jiménez-Bermúdez S, Pliego-Alfaro F, Quesada MA, Mercado JA (2000) *Agrobacterium* cells as microprojectile coating: a novel approach to enhance stable transformation rates in strawberry. *Aust J Plant Physiol* 27:1093–1100
- Cordero de Mesa M, Santiago-Doménech N, Pliego-Alfaro F, Quesada MA, Mercado JA (2004) The *CaMV* 35S promoter is highly active on floral organs and pollen of transgenic strawberry plants. *Plant Cell Rep* 23:32–38
- Dale A, Sjulín TM (1990) Few cytoplasm contribute to North American strawberry cultivars. *HortScience* 25:1341–1342
- du Plessis HJ, Brand RJ, Glyn-Woods C, Goedhart MA (1997) Efficient genetic transformation of strawberry (*Fragaria* × *ananassa* Duch.) cultivar Selekt. *Acta Hort* 447:289–294
- Dziadczyk P, Bolibok H, Tyrka M, Horthyński JA (2003) In vitro selection of strawberry (*Fragaria* × *ananassa* Duch.) clones tolerant to salt stress. *Euphytica* 132:49–55
- El Mansouri I, Mercado JA, Valpuesta V, López-Aranda JM, Pliego-Alfaro F, Quesada MA (1996) Shoot regeneration and *Agrobacterium*-mediated transformation of *Fragaria vesca* L. *Plant Cell Rep* 15:642–646
- Faedi W, Mourgues F, Rosati C (2002) Strawberry breeding and varieties: situation and perspectives. *Acta Hort* 567:51–59
- Finstad K, Martin RR (1995) Transformation of strawberry for virus resistance. *Acta Hort* 385:86–90
- George EF (1996) Plant propagation by tissue culture. Part 2. Exegetics, Edington
- Graham J (2005) *Fragaria* strawberry. In: Litz RE (ed) *Biotechnology of fruit and nut crops*. CABI, Wallingford, Oxon, pp 456–474
- Graham J, McNicol RJ, Greig K (1995) Towards genetic based insect resistance in strawberry using the cowpea trypsin inhibitor gene. *Ann Appl Biol* 127:163–173



- Graham J, Gordon SC, McNicol RJ (1997) The effect of the CpTi gene in strawberry against attack by vine weevil (*Otiorhynchus sulcatus* F. Coleoptera: Curculionidae). *Ann Appl Biol* 131:133–139
- Graham J, Gordon SC, Smith K, McNicol RJ, McNicol JW (2002) The effect of the cowpea trypsin inhibitor in strawberry on damage by vine weevil under field conditions. *J Hortic Sci Biotechnol* 77:33–40
- Gruchala A, Korbin M, Zurawicz E (2004) Conditions of transformation and regeneration of 'Induka' and 'Elista' strawberry plants. *Plant Cell Tissue Organ Cult* 79:153–160
- Hammerschlag F, Garcés S, Koch-Dean M, Ray S, Lewers K, Maas J, Smith BJ (2006) In vitro response of strawberry cultivars and regenerants to *Colletotrichum acutatum*. *Plant Cell Tissue Organ Cult* 84:255–261
- Hancock JF (1990) Ecological genetics of natural strawberry species. *HortScience* 25:869–871
- Hancock JF, Luby JJ, Dale A, Callow PW, Serce S, El-Shiek A (2002). Utilizing wild *Fragaria virginiana* in strawberry cultivar development: inheritance of photoperiod sensitivity, fruit size, gender, female fertility and disease resistance. *Euphytica* 126:177–184
- Hannum SM (2004) Potential impact of strawberries on human health: a review of the science. *Crit Rev Food Sci Nutr* 44:1–17
- Harpster MH, Brummell DA, Dunsmuir P (1998) Expression analysis of a ripening-specific, auxin-repressed endo-1,4- $\beta$ -glucanase gene in strawberry. *Plant Physiol* 118:1307–1316
- Haymes KM, Davis TM (1998) *Agrobacterium*-mediated transformation of 'Alpine' *Fragaria vesca*, and transmission of transgenes to R1 progeny. *Plant Cell Rep* 17:279–283
- Haymes KM, Van de Weg WE, Arens P, Maas JL, Vosman B, Den Nijs APM (2000). Development of SCAR markers linked to a *Phytophthora fragariae* resistance gene and their assessment in European and North American strawberry genotypes. *J Am Soc Hortic Sci* 125:330–339
- Houde M, Dallaire S, N'Dong D, Sarhan F (2004) Over-expression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves. *Plant Biotechnol J* 2:381–387
- Iannetta PPM, Medina-Escobar N, Ross HA, Souleyre EJE, Hancock RD, Witte C-P, Davies HV (2004) Identification, cloning and expression analysis of strawberry (*Fragaria*  $\times$  *ananassa*) mitochondrial citrate synthase and mitochondrial malate dehydrogenase. *Physiol Plant* 121:15–26
- James DJ, Passey AJ, Barbara DJ (1990) *Agrobacterium*-mediated transformation of the cultivated strawberry (*Fragaria*  $\times$  *ananassa* Duch.) using disarmed binary vectors. *Plant Sci* 69:79–94
- James DJ, Passey AJ, Eastbrook MA, Solomon MG, Barbara DJ (1992) Progress in the introduction of transgenes for pest and disease resistance into strawberries. *Phytoparasitica* 20:83–87
- Jiménez-Bermúdez S, Redondo-Nevado J, Muñoz-Blanco J, Caballero JL, López-Aranda JM, Valpuesta V, Pliego-Alfaro F, Quesada MA, Mercado JA (2002) Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiol* 128:751–759
- Jones OP, Waller BJ, Beech MG (1988) The production of strawberry plants from callus cultures. *Plant Cell Tissue Organ Cult* 12:235–241
- Lazarus CM, Macdonald H (1996) Characterization of a strawberry gene for auxin-binding protein, and its expression in insect cells. *Plant Mol Biol* 31:267–277
- Liu ZR, Sanford JC (1988) Plant regeneration by organogenesis from strawberry leaf and runner tissue. *HortScience* 23:1057–1059
- Llop-Tous I, Domínguez-Puigjaner E, Palomer X, Vendrell M (1999) Characterization of two divergent endo- $\beta$ -1,4-glucanase cDNA clones highly expressed in the nonclimacteric strawberry fruit. *Plant Physiol* 119:1415–1421
- Llop-Tous I, Domínguez-Puigjaner E, Vendrell M (2002) Characterization of a strawberry cDNA clone homologous to calcium-dependent protein kinases that is expressed during fruit ripening and affected by low temperature. *J Exp Bot* 53:2283–2285
- López-Aranda JM, Pliego-Alfaro F, López-Navidad I, Barceló-Muñoz M (1994) Micropropagation of strawberry (*Fragaria*  $\times$  *ananassa* Duch.). Effect of mineral salts, benzyladenine levels and number of subcultures on the in vitro and field behaviour of the obtained microplants and the fruiting capacity of their progeny. *J Hortic Sci* 69:625–637
- Maas JL (1998) Compendium of strawberry diseases. APS Press, St Paul



- Manning K (1994) Changes in gene expression during strawberry fruit ripening and their regulation by auxin. *Planta* 194:62–68
- Manning K (1998) Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. *Planta* 205:622–631
- Marta AE, Camadro EL, Díaz-Ricci JC, Castagnaro AP (2004) Breeding barriers between the cultivated strawberry, *Fragaria* × *ananassa*, and related wild germplasm. *Euphytica* 136:139–150
- Martinelli A, Gaiani A, Cella R (1997) *Agrobacterium*-mediated transformation of strawberry cultivar Marmolada® onebar\*. *Acta Hort* 439:169–173
- Martinez GA, Chaves AR, Civello PM (2004)  $\beta$ -xylosidase activity and expression of a  $\beta$ -xylosidase gene during strawberry fruit ripening. *Plant Physiol Biochem* 42:89–96
- Martinez M, Abraham Z, Gambardella M, Echaide M, Carbonero P, Diaz I (2005) The strawberry gene *Cyf1* encodes a phytocystatin with antifungal properties. *J Exp Bot* 56:1821–1829
- Mathews H, Wagoner W, Kellogg J, Bestwick R (1995) Genetic transformation of strawberry: stable integration of a gene to control biosynthesis of ethylene. *In Vitro Cell Dev Biol-Plant* 31:36–43
- Mathews H, Dewey V, Wagoner W, Bestwick RK (1998) Molecular and cellular evidence of chimaeric tissues in primary transgenics and elimination of chimaerism through improved selection protocols. *Transgenic Res* 7:123–129
- Medina-Escobar N, Cárdenas J, Moyano E, Caballero JL, Muñoz-Blanco J (1997) Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. *Plant Mol Biol* 34:867–877
- Medina-Escobar N, Cardenas J, Muñoz-Blanco J, Caballero JL (1998) Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding to a mRNA for a low-molecular-weight-heat-shock protein. *Plant Mol Biol* 36:33–42
- Mehli L, Schaart JG, Kjellens TD, Tran DH, Salentijn EMJ, Schouten HJ, Iversen T-H (2004) A gene encoding a polygalacturonase-inhibiting protein (PGIP) shows developmental regulation and pathogen-induced expression in strawberry. *New Phytol* 163:99–110
- Mezzetti B, Landi L, Scortichini L, Rebori A, Spena A, Pandolfini T (2002) Genetic engineering of parthenocarpic fruit development in strawberry. *Acta Hort* 567:101–104
- Mezzetti B, Landi L, Pandolfini T, Spena A (2004) The *defH9-iaaM* auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. *BMC Biotechnol* 4:4
- Miller AR, Chandler CK (1990) Plant regeneration from excised cotyledons of mature strawberry achenes. *HortScience* 25:569–571
- Monticelli S, Gentile A, Damiano C (2002) Regeneration and *Agrobacterium*-mediated transformation in stipules of strawberry. *Acta Hort* 567:105–107
- Morgan A, Baker CM, Chu JSF, Lee K, Crandall BA, Jose L (2002) Production of herbicide tolerant strawberry through genetic engineering. *Acta Hort* 567:113–115
- Moyano E, Portero-Robles I, Medina-Escobar N, Valpuesta V, Muñoz-Blanco J, Caballero JL (1998) A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. *Plant Physiol* 117:711–716
- Moyano E, Encinas-Villarejo S, López-Ráez JA, Redondo-Nevado J, Blanco-Portales R, Bellido ML, Sanz C, Caballero JL, Muñoz-Blanco J (2004) Comparative study between two strawberry pyruvate decarboxylase genes along fruit development and ripening, post-harvest and stress conditions. *Plant Sci* 166:835–845
- Nam Y-W, Tichit L, Leperlier M, Cuerq B, Marty I, Lelièvre J-M (1999) Isolation and characterization of mRNAs differentially expressed during ripening of wild strawberry (*Fragaria vesca* L.) fruits. *Plant Mol Biol* 39:629–636
- Ndong C, Ouellet F, Houde M, Sarhan F (1997) Gene expression during acclimation in strawberry. *Plant Cell Physiol* 38:863–870
- Nehra NS, Stushnoff C, Kartha KK (1989) Direct shoot regeneration from strawberry leaf disks. *J Am Soc Hortic Sci* 114:1014–1018

- Nehra NS, Stushnoff C, Kartha KK (1990a) Regeneration of plants from immature leaf-derived callus of strawberry (*Fragaria × ananassa*). Plant Sci 66:119–126
- Nehra NS, Chibbar RN, Kartha KK, Datla RSS, Crosby WL, Stushnoff C (1990b) Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disk regeneration system. Plant Cell Rep 9:293–298
- Nyman M, Wallin A (1988) Plant regeneration from strawberry (*Fragaria × ananassa*) mesophyll protoplasts. J Plant Physiol 133:375–377
- Nyman M, Wallin A (1992) Transient gene expression in strawberry (*Fragaria × ananassa* Duch.) protoplasts and the recovery of transgenic plants. Plant Cell Rep 11:105–108
- Owen HR, Miller AR (1996) Haploid plant regeneration from anther cultures of three North American cultivars of strawberry (*Fragaria × ananassa* Duch.). Plant Cell Rep 15:905–909
- Owens CL, Thomashow MF, Hancock JF, Iezzoni AF (2002) CBF1 orthologs in sour cherry and strawberry and the heterologous expression of CBF1 in strawberry. J Am Soc Hortic Sci 127:489–494
- Passey AJ, Barrett KJ, James DJ (2003) Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria × ananassa* Duch.) using a range of explant types. Plant Cell Rep 21:397–401
- Perkins-Veazie P (1995) Growth and ripening of strawberry fruit. Hortic Rev 17:267–297
- Pliego-Alfaro F, Jiménez-Bermúdez S, Muñoz-Blanco J, Caballero-Repullo JL, Trainotti L, Casadoro G, Valpuesta V, Barceló-Muñoz M, Cordero de Mesa M, Quesada-Felice MA, Mercado-Carmona JA (2002) Effects of pectate lyase and cellulase genes, in antisense expression, on modification of strawberry fruit softening. Proc 10th IAPTC&B (International Association for Plant Tissue and Biotechnology) Congr, Florida, p 101-A
- Pritts M (2002) Growing strawberries, healthy communities, strong economies and clean environments: what is the role of researcher? Acta Hort 567:411–417
- Puite K, Schaart J (1998) *Agrobacterium*-mediated transformation of the apple cultivars Gala, Golden delicious and Elstar, and the strawberry cultivars Gariguette, Polka and Elsanta. Acta Hort 484:547–556
- Redondo-Nevado J, Moyano E, Medina-Escobar N, Caballero JL, Muñoz-Blanco J (2001) A fruit-specific and developmentally regulated endopolygalacturonase gene from strawberry (*Fragaria × ananassa* cv. Chandler). J Exp Bot 52:1941–1945
- Ricardo VG, Coll Y, Castagnaro A, Diaz Ricci JC (2003) Transformation of a strawberry cultivar using a modified regeneration medium. HortScience 38:277–280
- Roskopf E (1999) Report of the berry working group (Strawberry, Raspberry/Blackberry, Blueberry). In: Traynor PL, Westwood JH (eds) Ecological effects of pest resistance genes in managed ecosystems. Information Systems for Biotechnology, Blacksburg, pp 67–72, <http://www.isb.vt.edu>
- Rugienius R, Stanys V (2001) In vitro screening of strawberry plants for cold resistance. Euphytica 122:269–277
- Rugini E, Orlando R (1992) High efficiency shoot regeneration from calluses of strawberry (*Fragaria × ananassa* Duch.) stipules of in vitro shoot cultures. J Hortic Sci 67:577–582
- Salentijn EMJ, Aharoni A, Schaart JG, Boone MJ, Krens FA (2003) Differential gene expression analysis of strawberry cultivars that differ in fruit-firmness. Physiol Plant 118:571–578
- Schaart JG, Salentijn EMJ, Krens FA (2002) Tissue-specific expression of the  $\beta$ -glucuronidase reporter gene in transgenic strawberry (*Fragaria × ananassa*) plants. Plant Cell Rep 21:313–319
- Schaart JG, Krens FA, Pelgrom KTB, Mendes O, Rouwendal GJA (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J 2:233–240
- Sesmero R, Quesada MA, Mercado JA (2007) Antisense inhibition of pectate lyase gene expression in strawberry fruit: characteristics of fruits processed into jam. J Food Eng 79:194–199
- Shestibratov KA, Dolgov SV (2005) Transgenic strawberry plants expressing a thaumatin II gene demonstrate enhanced resistance to *Botrytis cinerea*. Sci Hort 106:177–189
- Sjulin TM (2003) The North American small fruit industry 1903–2003. II. Contributions of public and private research in the past 25 years and a view to the future. HortScience 38:960–967

- Sjulin TM, Dale A (1987) Genetic diversity of North American strawberry cultivars. *J Am Soc Hortic Sci* 112:375–385
- Sorvari S, Ulvinen S, Hietaranta T, Hiirsalmi H (1993) Preculture medium promotes direct shoot regeneration from micropropagated strawberry leaf disks. *HortScience* 28:55–57
- Sowik I, Bielenin A, Michalczyk (2001) In vitro testing of strawberry resistance to *Verticillium dahliae* and *Phytophthora cactorum*. *Sci Hort* 88:31–40
- Spolaore S, Trainotti L, Pavanello A, Casadoro G (2003) Isolation and promoter analysis of two genes encoding different endo- $\beta$ -1,4-glucanases in the non-climacteric strawberry. *J Exp Bot* 54:271–277
- Tian M, Gu Q, Zhu M (2003) The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. *Plant Sci* 165:701–707
- Toyoda H, Horikoshi K, Yamano Y, Ouchi S (1991) Selection for *Fusarium* wilt disease resistance from regenerants derived from leaf callus of strawberry. *Plant Cell Rep* 10:167–170
- Trainotti L, Spolaore S, Pavanello A, Baldan B, Casadoro G (1999) A novel E-type endo- $\beta$ -1,4-glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits. *Plant Mol Biol* 40:323–332
- Trainotti L, Spinello R, Piovan A, Spolaore S, Casadoro G (2001)  $\beta$ -Galactosidases with a lectin-like domain are expressed in strawberry. *J Exp Bot* 52:1635–1645
- USDA (2004). The U.S. and world situation: strawberries. <http://www.fas.usda.gov/http/hort.circular/2004/charts%20circular/toc.htm>
- Vellicce GR, Díaz Ricci JC, Hernández L, Castagnaro AP (2006) Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene *ch5B* in strawberry. *Transgenic Res* 15:57–68
- Wang DY, Wergin WP, Zimmerman RH (1984) Somatic embryogenesis and plant regeneration from immature embryos of strawberry. *HortScience* 19:71–72
- Wang J, Ge H, Peng S, Zhang H, Chen P, Xu J (2004) Transformation of strawberry (*Fragaria ananassa* Duch.) with late embryogenesis abundant protein gene. *J Hortic Sci Biotechnol* 79:735–738
- Watt K, Graham J, Gordon SC, Woodhead M, McNicol RJ (1999) Current and future transgenic control strategies to vine weevil and other insect resistance in strawberry. *J Hortic Sci Biotechnol* 74:409–421
- Wawrzynczak D, Sowik I, Michalczyk L (2000) *Agrobacterium*-mediated transformation of five strawberry genotypes. *J Fruit Ornam Plant Res* 8:1–8
- Wawrzynczak D, Michalczyk L, Sowik I (2005) Modification in indole-3-acetic acid metabolism, growth and development of strawberry through transformation with maize IAA-glucose synthase gene (*iaglu*). *Acta Physiol Plant* 27:19–27
- Woolley LC, James DJ, Manning K (2001) Purification and properties of an endo- $\beta$ -1,4-glucanase from strawberry and down-regulation of the corresponding gene, *cel1*. *Planta* 214:11–21
- Yonghua Q, Shanglong Z, Asghar S, Lingxiao Z, Qiaoping Q, Kunsong C, Changjie X (2005) Regeneration mechanism of Toyonoka strawberry under different color plastic films. *Plant Sci* 168:1425–1431
- Yubero-Serrano EM, Cordero de Mesa M, de los Santos B, Romero F, Rey M, Llobell A, Mercado JA, Pliego-Alfaro F, Muñoz-Blanco J, Caballero JL (2002) Obtención de plantas transgénicas de fresa con genes antifúngicos de *Trichoderma* como alternativa a tratamientos químicos no respetuosos con el medio ambiente. *Proc Congr Nac Biotecnol*, Sevilla, p 143
- Yubero-Serrano EM, Moyano E, Medina-Escobar N, Muñoz-Blanco J, Caballero JL (2003) Identification of a strawberry gene encoding a non-specific lipid transfer protein that responds to ABA, wounding and cold stress. *J Exp Bot* 54:1865–1877
- Zhao Y, Qingzhong L, Davis RE (2004) Transgene expression in strawberries driven by a heterologous phloem-specific promoter. *Plant Cell Rep* 23:224–230
- Zhou Y-H, Guo D-P, Zhu Z-J, Qian Q-Q (2005) Effects of in vitro rooting environments and irradiance on growth and photosynthesis of strawberry plantlets during acclimatization. *Plant Cell Tissue Organ Cult* 81:105–108

## I.13 Kiwifruit

R.G. ATKINSON and E.A. MACRAE<sup>1</sup>

### 1 Introduction

Kiwifruit are a national icon in New Zealand. Nowhere else in the world do kiwifruit appear on postage stamps, can you buy kiwifruit-shaped soft toys, and can tourists take a drive in a kiwi-kart through an entire kiwifruit theme park. Yet kiwifruit are not native to New Zealand. The centre of diversity for the genus *Actinidia* is South Eastern Asia, in particular the Yangtze Valley of China. Kiwifruit's association with New Zealand only started in the early twentieth century when a missionary returning from China brought a small handful of seed to New Zealand. Several cultivars were selected by amateur horticulturalists in the 1930s and the New Zealand export industry was developed around fruit of the *A. deliciosa* cultivar Hayward in the 1960s (Ferguson et al. 1996). During the 1980s, breeding programs were established in New Zealand using additional germplasm imported from China. The first internationally successful product from the New Zealand breeding program was a large gold-fruited *A. chinensis* cultivar, released commercially in 1998 as ZESPRI™ GOLD. New products currently being released are sweet-flavored *A. arguta* selections and further *A. deliciosa* and *A. chinensis* selections that are defined by specific market- and production-related targets (Ferguson et al. 1996; Seal 2003). It is not surprising, given its history of commercial development in New Zealand, that the drive to apply biotechnology to kiwifruit is also being driven by research within New Zealand (n.b. kiwifruit will be used here as a generic term to describe all species of *Actinidia*).

This chapter provides an update on the economic importance of kiwifruit in the context of other fruit crops and examines the 'genetic potential' of the *Actinidia* genus. It summarizes advances in molecular biology and transformation technology that have been applied to kiwifruit, with special reference to the development of expressed sequence tags (EST) databases and a functional genomics program at HortResearch in New Zealand. Finally it describes some of the practical applications that biotechnology provides kiwifruit.

---

<sup>1</sup> The Horticulture and Food Research Institute of New Zealand Ltd., Mt Albert Research Centre, Private Bag 92169, Auckland, New Zealand, e-mail: ross.atkinson@hortresearch.co.nz

## 2 Economic Importance and Genetic Potential

As a newly commercialized fruit crop, kiwifruit is a small but growing player in the world fruit market, representing 0.25% of total production compared with citrus (23%), banana (15%), grape (14%) and apple (13%). The majority of the crop is sold in the fresh market, with only a very small proportion being processed into products such as juice, jam and wine. In the previous decade, production in all major fruit categories increased more than world population growth, except for kiwifruit and grapes. Many of the minor deciduous, citrus and tropical fruits that compete closely with kiwifruit for market share had the highest rate of growth (Belrose, Inc. 2004). Although kiwifruit has been classified in the specialty fruit category, it should now rightly be considered as a fruit commodity. The top four kiwifruit-producing nations of Italy, New Zealand, China and Chile dominate world production with 30, 21, 18 and 13% of the total crop respectively. Total world production exceeds 1.2 million tons per year. Significant plantings are also found in France, Japan, Greece, the United States, Iran and Spain. The most significant increases (>20%) in planted area during the previous decade occurred in China, Iran and Spain, whilst decreases (>15%) were recorded in Greece, Japan and South Korea (Belrose, Inc. 2004). A characteristic feature of the kiwifruit industry is low production in most of the rich countries (e.g. Germany, the United Kingdom) to which the fruit is exported. According to Belrose, Inc., "These countries have little or no incentive to assist the kiwifruit industry in research, promotion or marketing in order to stimulate total consumption at the expense of other fruits" (Belrose, Inc. 2004).

The potential of kiwifruit for development via traditional breeding and biotechnology is high. There are >70 *Actinidia* species, but only three, *A. deliciosa*, *A. chinensis* and *A. arguta*, have been commercially exploited to date (Ferguson 1999). Breeding programs in New Zealand and Italy are directed primarily at producing *A. deliciosa* and *A. chinensis* varieties with large fruit size, good flavor, novel flesh color, variations in harvest period (both early and late), improved yield and growth habit, and hermaphroditism (Ferguson et al. 1996). Longer-term breeding objectives include the development of novel high value fruit with increased consumer health and convenience attributes e.g. peelable skins, edible fruit skins, and fruit that change color as they ripen (Seal 2003). The use of molecular biology and functional genomics allows for a greater understanding of the processes that control these fruit characters, and the chance to manipulate desirable and undesirable characters by protein over-expression/down-regulation or through marker assisted breeding.

The extensive kiwifruit germplasm resources available in China and New Zealand also present an opportunity for exploitation of non-fruit-based products. Kiwifruit flowers and fruit contain many unique and interesting flavor and aroma compounds, and corresponding genetic pathways that have potential in bioproduction systems, whilst the leaves, stems and roots contain compounds with potential for exploitation as nutraceuticals or in medicine.

Kiwifruit contain nutrients known to promote human health, including magnesium, potassium, folate, and vitamins C and E. The fruit is low in fat, and a good source of dietary fibre and phytochemicals (Ferguson and Ferguson 2003). Below is a list of some of the desirable characters and compounds of interest in kiwifruit that can be exploited using biotechnology.

## 2.1 Health and Nutritional Components

- Fruit of *Actinidia* species are high in vitamin C which is recognized as having antioxidant and immune-stimulation properties (Ferguson and Ferguson 2003; Nishiyama et al. 2004). *A. kolomikta* and *A. latifolia* are reported to contain in excess of 1,000 mg vitamin C per 100 g fresh weight (Ferguson 1990). Genes in the ascorbic acid biosynthetic pathway in *Actinidia* have been targeted for isolation and manipulation (Laing et al. 2004a,b).
- Hayward fruit have beneficial laxative properties. One kiwifruit per 30 kg bodyweight for 3 weeks, in adults over 60, enhanced all tested measures of laxation (Rush et al. 2002). Consumption of two to three kiwifruit per day for 28 days lowers blood triglyceride levels and reduces platelet aggregation response to collagen and ADP (Duttaroy and Jorgensen 2004). Consumption of kiwifruit enhances antioxidant status in lymphocytes and stimulates DNA repair (Collins et al. 2003).
- *A. deliciosa* fruit contains a thaumatin-like protein which inhibits HIV-1 reverse transcriptase and inhibits growth of *Botrytis cinerea* (Wurms et al. 1999; Wang and Ng 2002).
- *A. chinensis* extracts show anti-mutagenic activity against picrolonic acid-induced mutation, and can inhibit the mutagenicity of benzopyrene completely (Lee and Lin 1988). Leaf extracts contain flavonoids such as rutin, esperidin, quercetin, apigenin and canferol which show antioxidant, antimicrobial, anti-inflammatory and other effects on eukaryotic cells (Basile et al. 2000). Fruit extracts show selective cytotoxic activity against human oral tumor cell lines, anti-HIV activity, radical generation, O<sub>2</sub>-scavenging activity and antibacterial activity (Motohashi et al. 2002).
- *A. arguta* extracts contain chlorophyll derivatives that induce apoptotic cell death in human Jurkat T cells (Park et al. 2000), whilst *A. arguta* stems contain immunopharmacological polysaccharides (Hou et al. 1995), and catechin and epicatechin which act as bone marrow cell proliferation promoting compounds (Takano et al. 2003).
- *A. polygama* fruit contain a compound for treating pain, rheumatic arthritis and inflammation (Kim et al. 2003) and the juice strongly induced differentiation of HL-60 cells to monocyte/macrophage characteristics in a concentration-dependent manner (Yoshizawa et al. 2000a). Juice also strongly inhibited the proliferation of all cancer cell lines examined, yet the juice was substantially less cytotoxic toward normal human cell lines



(Yoshizawa et al. 2000b). Fruit galls of *A. polygama* produce triterpenoids that can be used as an analgesic (Sashida et al. 1992, 1994).

- Some constituents of kiwifruit are less desirable. Fruit have been shown to be capable of causing allergic reactions, particularly in young children (Lucas et al. 2004). Cross reactivity occurs with allergens in latex (Moller et al. 1998) and birch pollen (Voitenko et al. 1997). Some of the allergenic proteins have been identified, e.g. thaumatin-like protein (Gavrovic-Jankulovic et al. 2002) and actinidin (Pastorello et al. 1998). Fruit can cause dermatitis (Zina and Bundino 1983). Fruit contain raphide crystals of calcium oxalate that contribute to the 'catch' sensation experienced by some consumers (Perera and Hallett 1991; Walker and Prescott 2003).

## 2.2 Flavor, Fragrance and Color

- Fruit of many *Actinidia* species contain interesting flavor and aroma compounds such as novel esters, terpenes and aldehydes (Young et al. 1995; Gilbert et al. 1996; Jordan et al. 2002). *A. deliciosa* 'Hayward' are described by consumers as having fresh, sweet and acid flavors, ZESPRI™ GOLD as having sweet, fruity flavors (Jaeger et al. 2002), whilst fruit of recent *A. arguta* selections have flavors reminiscent of ripe strawberries, bananas, over-ripe pears or rhubarb (Williams et al. 2003).
- Mapping populations segregate for acid and sugar characters that have been shown to be important in consumer perception of flavor (Marsh et al. 2003, 2004; Cheng et al. 2004). In particular, flavor acceptability increases with increasing soluble solids concentration (Rossiter et al. 2000). Genes involved in sugar metabolism (e.g. sucrose phosphate synthase and sucrose synthase) have been targeted, isolated and manipulated (Langenkämper et al. 1998; Fung et al. 2003).
- Flowers of many *Actinidia* species, such as *A. arguta* (Matich et al. 2003), are rich in volatile compounds including terpenes, esters, interesting sulfur compounds and unusual ketones.
- Gene targets of interest in flavor and aroma include terpene synthase genes (Green et al. 2004), alcohol acetyl transferases, methyl transferases, lipooxygenases and dehydrogenases.
- Flesh color in kiwifruit includes light and dark green, yellow, orange, red, purple or green/yellow with red coloration in the center. Skin colors include green, brown, reddish and purple (Ferguson et al. 1996). The carotenoid contents of *A. deliciosa* green fruit and *A. chinensis* gold fruit are similar; however, in gold fruit chlorophyll content is reduced as the fruit ripens (McGhie and Ainge 2002). Genes involved in carotenoid and anthocyanin biosynthesis, and chlorophyll degradation are targets for isolation.



### 2.3 Convenience and Food Safety Targets

- *Actinidia* fruit have many convenience and food safety attributes that are becoming increasingly important drivers in consumer fruit purchasing.
- *A. polygama* fruit change color from green to yellow on ripening.
- *A. arguta* produce bite-sized fruit that can be eaten as a snack. The fruit are hairless and the skin edible (Williams et al. 2003).
- *A. eriantha* accessions have a peelable skin.

### 2.4 Fruit Production and Storage Characteristics

- *A. deliciosa* and *A. chinensis* varieties are generally large fruited (80–100 g) and have good storage characteristics. *A. deliciosa* fruit can be stored for 4–6 months at  $0 \pm 0.5^{\circ}\text{C}$  (McDonald 1990), an important selection criteria for varieties grown in New Zealand given the distance to export markets. Most other *Actinidia* species have significantly shorter storage lives (e.g. Hassall et al. 1998; White et al. 2005).
- Fruit softening occurs independently of endogenous ethylene production, and flavor and aroma production occurs after fruit softening (MacRae and Redgwell 1992). Genes in the ethylene biosynthetic pathway are targets for genetic manipulation investigating the effects on the flavor/aroma profile and post-harvest storage life.

### 2.5 Other Targets

- Axillary bud development and subsequent flower differentiation in kiwifruit are spread over two growing seasons. The two phases are separated by a long period of morphological inactivity. Genes that control floral evocation are the subject of molecular investigation with a view to better understanding how to control the downstream harvest window (Walton et al. 2001). Proline accumulates in breaking buds and proline biosynthetic genes have been implicated in the evocation process (Walton et al. 1998). Mutants in floral development have been documented, including the *A. chinensis*  $\times$  *A. eriantha* hybrid ‘Jianshanjiao’ which flowers three to five times a year, the *A. deliciosa* agamous-like homeotic mutant ‘Pukekohe dwarf’, and *A. eriantha* vines with flowers that do not open.
- Cold hardiness traits are found in *A. arguta* and *A. kolomikta*. *A. arguta* (also known as ‘hardy kiwi’) can withstand winter temperatures as low as  $-30^{\circ}\text{C}$  (Williams et al. 2003).
- Hermaphroditism is a trait that occurs in kiwifruit and is the subject of an intensive breeding effort at HortResearch. Programmed cell death has been implicated in male sterility of female *A. deliciosa* flowers (Coimbra et al. 2004). Hermaphroditic variants such as fruiting males and fertile pollen-containing females have been observed in several *Actinidia* species (Ferguson et al. 1996; Testolin et al. 2004).

### 3 Molecular Biology

#### 3.1 Background

Due to its recent domestication, our understanding of the genetics, physiology and biochemistry of kiwifruit is not as well developed as in other fruiting species. Furthermore, several features of kiwifruit make them less amenable to breeding and application of biotechnology than other crops. *Actinidia* species are extremely vigorous climbing or scrambling vines that may grow 4–5 m in a season; moreover, most have juvenile periods of 3–5 years and bear fruit only once per year. All *Actinidia* species are functionally dioecious. Thus, two selected female parents cannot be crossed directly and the breeding value of male parents can only be measured indirectly. The genus has a reticulate polyploid structure with diploids, tetraploids, hexaploids and octaploids (Ferguson et al. 1997). The basic chromosome number is  $x = 29$  with a diploid number of 58. Chromosomes are small and difficult to identify individually (Ferguson et al. 1996). Individual species are found as races of different ploidy, e.g. *A. chinensis* is found as both a diploid and a tetraploid; *A. arguta*, although generally tetraploid, is also found as a diploid, tetraploid or octaploid (Ferguson et al. 1997). Consideration of these ploidy differences is important when making interspecific crosses (Kobayashi et al. 2000; Hirsch et al. 2001). Biochemical and molecular studies must take into account the multiplicity of genes coding for a particular function (MacRae and Atkinson 2003).

Protocols for routine application of molecular biology and biochemistry techniques have needed to be developed for kiwifruit to overcome high fruit acidity, mucilaginous extracts and the presence of the cysteine protease actinidin. Today, protocols are available to obtain high quality RNA (e.g. Ledger and Gardner 1994; Langenkämper et al. 1998) and DNA (e.g. Janssen and Gardner 1993; Kobayashi et al. 1996), for use in Northern and Southern analysis (e.g. Kobayashi et al. 1996; Schröder et al. 1998; Chen et al. 1999), cDNA library production (e.g. Ledger and Gardner 1994; Langenkämper et al. 1998) and BAC library construction (Hilario et al. 2004), as well as semi-quantitative PCR (e.g. Wang et al. 2000; Fung et al. 2003) and quantitative PCR (HortResearch 2005). Protein extraction protocols from fruit and other tissues have also been developed and applied to enzymatic analysis (e.g. MacRae et al. 1992; Marquis and Bucheli 1994; Sugiyama et al. 1996; Laing et al. 2004b), western blotting (e.g. Langenkämper et al. 1998) and proteomics studies (HortResearch 2005).

#### 3.2 Gene Sequences Deposited in GenBank

As of October 2004, 439 entries for all *Actinidia* species were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/>). However, if sequences used for phylogenetic studies, such as mitochondrial and chloroplast genes/spacer regions are excluded, the number is only 71. Of these sequences, 44 are from

*A. deliciosa*, 24 from *A. chinensis*, 2 from *A. arguta* and 1 sequence is from *A. eriantha*. The majority of the sequences deposited are expressed in fruit, reflecting the economic importance of this tissue. Almost 50% of the genes are represented by only four families, namely, 1-aminocyclopropane-1-carboxylate (ACC) synthase (8 sequences), polygalacturonase (8), sucrose phosphate synthase (8) and actinidin (10). These four gene families are representative of research that has been carried out in kiwifruit to date.

ACC synthase (Whittaker et al. 1997), ACC oxidase (MacDiarmid and Gardner 1993), S-adenosyl-L-methionine synthase (Whittaker et al. 1995) and the ethylene receptor (GenBank entries AY464566, AY496451, AY496452) represent genes involved in the control of ethylene biosynthesis, a potential control point in kiwifruit ripening and post-harvest fruit management. Polygalacturonase (Atkinson and Gardner 1993; Wang et al. 2000), xyloglucan endotransglucosylase/hydrolase (Schröder et al. 1998) and polygalacturonase inhibitor protein (Simpson et al. 1995) are genes involved in the raft of cell wall modifications that occur during fruit ripening and impact on the texture and shelf life of fruit. Sucrose phosphate synthase (Langenkämper et al. 1998; Langenkämper et al. 2002) and sucrose synthase (Richardson et al. 2004) are genes involved in the control of carbohydrate metabolism that are important in determining fruit sweetness and flavor.

Actinidin genes (Praekelt et al. 1988; Podivinsky et al. 1989) encode a cysteine protease that can account for up to 50% of the soluble protein in fruit. Six different isozymes have been isolated from the fruit with slightly different enzyme properties (Sugiyama et al. 1996). The protein has been crystallized and the structure determined to 1.7 Å resolution (Baker 1980). The actinidin promoter has been sequenced (Keeling et al. 1990) and its fruit developmental regulation shown to be conserved in petunia (Lin et al. 1993). The gene was over-expressed in tobacco where it was catalytically active, but high expression was detrimental to growth (Paul et al. 1995). The protein has been implicated in allergenic responses to kiwifruit ingestion (Pastorello et al. 1998). Despite the high levels of protein found in fruit and its extensive characterization, the function of actinidin is still not known.

Multiple sequences appear in GenBank in two other areas of research: five genes differentially expressed early in fruit development (Ledger and Gardner 1994) and four genes involved in floral differentiation (Walton et al. 1998, 2001).

### 3.3 EST Databases at HortResearch

HortResearch has developed a proprietary database of >130,000 expressed sequence tags (ESTs) to exploit the genetic potential of kiwifruit (HortResearch 2005). The ESTs were derived from 35 libraries that covered a range of tissues and *Actinidia* species. The sequences were parsed through two rounds of contig building from which a set of kiwifruit 'unigenes' was derived. ESTs and unigenes were BLAST and motif searched and, where possible, automatically

**Table 1.** Number of HortResearch ESTs by tissue type and species

Number of ESTs by tissue type		Number of ESTs by species	
Cell culture	4,759	<i>A. arguta</i>	7,370
Fruit	38,992	<i>A. chinensis</i>	47,568
Leaf	17,866	<i>A. deliciosa</i>	59,163
Petal	16,019	<i>A. eriantha</i>	13,030
Roots	4,808	Other <sup>a</sup>	7,185
Shoot buds	50,841	–	
Stem	1,031	–	

<sup>a</sup> *A. polygama*, *A. setosa*, *A. indochinensis*  
and *A. hemsleyana* × *A. eriantha*

annotated to assign a predicted gene class or function. A summary of the number of ESTs derived from the eight *Actinidia* species and the seven major tissue types used in library construction is given in Table 1.

Libraries were made to reflect the potential for gene discovery from *Actinidia* species. Hence, a wide sampling across different *Actinidia* species was undertaken. Target areas included secondary metabolites, particularly in order to discover flavor and fragrance pathways and pathways for health-related metabolites, vitamin C and oxalate pathways, bud dormancy release, skin peelability and edibility, and polysaccharide synthetic and degradative processes. Libraries were also constructed from a spread of tissues to obtain genes for use in microarray analysis. These libraries were primarily derived from *A. chinensis* diploid genotypes to reduce the impact of ploidy. In *A. deliciosa*, the principal targets were genes involved in breaking bud dormancy and regulation of flowering. In some instances, libraries were subtracted to enrich for genes of interest before sequencing, e.g. petal libraries across various species, some fruit libraries were normalized against actinidin, and for several libraries >10,000 ESTs were sequenced, depending on the degree of sequence novelty.

### 3.4 Microarray Technology

HortResearch has developed a 17,472 gene microarray of kiwifruit genes which will allow monitoring of genome-wide expression patterns in kiwifruit (HortResearch 2005). The arrays consist of unique 50-mer oligonucleotides designed to the most 3' end of each kiwifruit unigene. Unigenes on the array were derived from *A. chinensis* (8957 sequences), *A. deliciosa* (6606), *A. eriantha* (978) and *A. arguta* (479), whilst all other *Actinidia* species contributed 276 genes. *A. chinensis* was chosen as the principal species to contribute sequences to the array as it is a diploid, closely related to *A. deliciosa* (Crowhurst et al. 1990; Atkinson et al. 1997), and contributed at least one (possibly two) of its genomes to the hexaploid *A. deliciosa* (Yan et al. 1997). *A. chinensis* is also the source of the first kiwifruit mapping populations (Testolin et al. 2001; Fraser

et al. 2004). The expectation was that most genes expressed in the *A. chinensis* genome should also hybridize with those expressed in *A. deliciosa*. Labeled RNA from *A. chinensis* has been shown to hybridize to oligonucleotides that correspond to genes from all *Actinidia* species represented on the array, indicating that cross-species studies within *Actinidia* are feasible (HortResearch 2005). Currently, the arrays are being used to monitor gene expression relating to the targets described in Section 3.3 and during key developmental processes, e.g. bud break and fruit development and ripening.

### 3.5 BAC Libraries and Genome Comparisons

Comparative mapping is a powerful approach for analyzing genomes of divergent species since gene linkage is often conserved over evolutionary time (microsynteny). A BAC library from *A. chinensis* was commercially prepared by Bio S&T, Quebec, Canada, for HortResearch. The library covers the *A. chinensis* genome at least 7.6 times and clones contain large inserts (>71 kbp) and very low amounts of DNA from organelles (Hilario et al. 2004). The library has been printed on high density nylon filters and is being used to construct a BAC sequence-ready map around the sex locus in *A. chinensis* (Hilario et al. 2004). This resource will allow BAC clones to be assigned to hundreds of the ESTs in the HortResearch database and will form the basis of future map-based cloning and genome sequencing efforts. The resource will also facilitate intra- and inter-specific genome comparisons in the *Actinidia* genus and will complement molecular phylogenetic approaches to evolution in the Actinidiaceae (Cipriani et al. 1998; Li et al. 2003; Chat et al. 2004).

## 4 Genetic Transformation and Functional Genomics

### 4.1 Genetic Transformation

Many tissue culture techniques, e.g. embryo rescue, somatic embryogenesis, haploid recovery, triploid recovery from endosperm, protoplast isolation and culture micropropagation and cryopreservation, have been successfully applied to kiwifruit (Oliveira and Fraser 2005). For genetic transformation, the requirement is for a system of plant regeneration through de novo shoot induction. This has been achieved for a range of *Actinidia* species including *A. deliciosa*, *A. chinensis*, *A. arguta*, *A. eriantha*, *A. polygama* and *A. kolomikta* (Nayak and Beyl 1987; Ferguson et al. 1996; Famiani et al. 1997). The tissues with the most organogenic potential have been leaf blades, stems and petioles, although other tissues including roots, hypocotyls and protoplasts have been used (reviewed in Ferguson et al. 1996).

Transformation systems have been developed for five *Actinidia* species (Table 2) using reporter genes such as neomycin phosphotransferaseII,  $\beta$ -glu-

**Table 2.** Development of transformation systems for kiwifruit

Species	<i>A. tumefaciens</i> strain	Tissue(s) transformed	Reference
<i>A. deliciosa</i>	LBA4404	Leaf, petiole, stem	Matsuta et al. (1990, 1993)
	–	Protoplasts	Oliveira et al. (1991) <sup>a</sup>
	EHA101	Hypocotyl, stem	Uematsu et al. (1991)
	A281, C58, LBA4404, EHA101	Leaf	Janssen and Gardner (1993)
	NIAES 1724 <sup>b</sup> ArM 123, IFO 14555, A5, A13 <sup>b</sup>	Hypocotyls Petioles	Yazawa et al. (1995) Yamakawa and Chen (1996)
<i>A. chinensis</i>	A281, C58	Leaf, petiole, stem	Fraser et al. (1995)
<i>A. arguta</i>	A281, C58	Leaf, petiole, stem	HortResearch (2005)
<i>A. eriantha</i>	LBA4404	Leaf	Wang et al. (2006)
<i>A. kolomikta</i>	Not described	Not described	Firsov and Dolgov (1997)

<sup>a</sup> Direct gene transfer using polyethylene glycol-mediated transfection; regenerated plants not reported

<sup>b</sup> *Agrobacterium rhizogenes* strains

curonidase and chloramphenicol acetyl transferase. Virulent and disarmed strains of *Agrobacterium* have been used to achieve genetic transformation, with the disarmed *Agrobacterium tumefaciens* strain LBA4404 being the most commonly utilized. Functional genomic studies have been carried out in four *Actinidia* species (*A. deliciosa*, *A. chinensis*, *A. arguta* and *A. kolomikta*) with genes obtained from other *Actinidia* species, grape, human, soybean and rice (see Table 3). The purpose of these studies is quite diverse, including the manipulation of fruit characters, production of bioactive compounds and attempts to alter plant development. A number of kiwifruit genes and promoters have also been functionally tested in other species for a range of different purposes (Table 4). There are two reports of transgenic *A. deliciosa* being grown to maturity and the transmission of transgenes to progeny plants (Rugini et al. 1997; Fung et al. 1998). Field trials of transgenic vines of *A. deliciosa* have been reported in Janssen and Gardner (1993) and Biasi et al (2003).

## 4.2 High Throughput Functional Genomics

A high throughput functional genomics platform to characterize kiwifruit genes from the EST database has been developed at HortResearch. This platform includes transient and transgenic expression in *Escherichia coli*, yeast, *Arabidopsis* and other model plant systems (HortResearch 2005). Significant improvements in the efficiency of transformation of *A. deliciosa* and *A. chinensis* have also been achieved (Wang et al. 2002) and a transformation system for *A. eriantha* has been developed (Wang et al. 2006). In both cases, the

**Table 3.** Functional genomics in kiwifruit

Species	Gene and source	Purpose	Reference
<i>A. deliciosa</i>	<i>rol</i> A, B, C from <i>A. rhizogenes</i>	Improve rooting	Rugini et al. (1991, 1997)
	Human epidermal growth factor	Production of bioactives	Kobayashi et al. (1996) <sup>a</sup>
	Soybean $\beta$ -1-3-endoglucanase	Increase disease resistance	Nakamura et al. (1999) <sup>a</sup>
	Rice <i>OSH1</i> homeobox gene	Alter plant development	Kusaba et al. (1999)
	<i>Vitis</i> stilbene gene	Increase disease resistance	Kobayashi et al. (2000)
	<i>A. deliciosa</i> P5CR; P5CS <sup>b</sup>	Alter proline synthesis	HortResearch (2005)
<i>A. chinensis</i>	<i>A. chinensis</i> SPS <sup>c</sup>	Alter sugar metabolism	Fung (2003)
	TYDV <sup>d</sup> promoter	Test promoter specificity	Fung (2003)
	<i>A. chinensis</i> actinidin	Identify function	HortResearch (2005)
	<i>A. chinensis</i> ACC oxidase <sup>e</sup>	Improve fruit storage	HortResearch (2005)
<i>A. arguta</i>	<i>A. chinensis</i> PG <sup>f</sup>	Improve fruit quality	HortResearch (2005)
<i>A. kolomikta</i>	<i>A. rhizogenes rol</i> C	Improved rooting	Dolgov (2004)
	Bt <sup>g</sup> toxin	Increase resistance to insects	
	Arctic flounder antifreeze protein	Increase cold tolerance	

<sup>a</sup> Incorrectly named *A. chinensis*

<sup>b</sup> P5CR = pyrroline-5-carboxylate reductase, P5CS = pyrroline-5-carboxylate synthase

<sup>c</sup> SPS = sucrose phosphate synthase

<sup>d</sup> TYDV = tobacco yellow dwarf virus

<sup>e</sup> ACC oxidase = 1-aminocyclopropane-1-carboxylic acid oxidase

<sup>f</sup> PG = polygalacturonase

<sup>g</sup> Bt = *Bacillus thuringiensis*

preference has been to produce plants using disarmed strains to remove the potential for co-integration of oncogenic plasmids found in virulent strains. Thirty-two constructs for genes involved in flavor and aroma biosynthesis transcriptional control, chromatin remodeling, ascorbic acid biosynthesis and cell wall modification have been transformed into kiwifruit in the last two years (HortResearch 2005). The bottleneck in this high throughput analysis has been the inability to field trial plants due to a moratorium on the release of genetically modified organisms into the environment in New Zealand. Consequently, protocols have been developed to obtain flowering and fruiting plants in standard containment glasshouses at the Mt Albert Research Centre in Auckland, New Zealand, and in specialized controlled environment containment facilities at the National Climate Laboratories in Palmerston North, New Zealand.



**Table 4.** Kiwifruit genes or promoters transformed into other plant species

Kiwifruit sequence	Host species	Purpose	Reference
Actinidin promoter	Petunia	Determine tissue specificity of promoter	Lin et al. (1993)
Actinidin	Tobacco	Determine N and C terminal protein targeting	Paul et al. (1995)
PG promoter	Tomato	Determine tissue specificity of promoter	Wang et al. (2000)
PGIP <sup>a</sup>	Apple	Increase antifungal potential of apple	Szankowski et al. (2003)
SPS promoter	Tobacco	Sugar and hormone responsiveness of promoter	Fung (2003)
GD synthase <sup>b</sup>	Arabidopsis	Alter volatile profile	Green et al. (2004)
XTH <sup>c</sup> promoter	Arabidopsis	Determine tissue specificity of promoter	HortResearch (2005)
SPS promoter	Arabidopsis	Tissue specificity, and sugar and hormone responsiveness of promoter	HortResearch (2005)
SPS promoter	Tobacco	Discover transcription factors activating promoter	HortResearch (2005)

<sup>a</sup> PGIP = polygalacturonase inhibitor protein

<sup>b</sup> GD synthase = germacrene-D synthase

<sup>c</sup> XTH = xyloglucan transglucosylase/hydrolase

## 5 Current and Future Practical Applications of Biotechnology

Biotechnology is currently being applied in kiwifruit breeding. The introgression of characters from one *Actinidia* species into another requires an understanding of the ploidy levels of the species involved. Flow cytometry is used to test the ploidy levels of selected parents (Ollitrault-Sammarcelli et al. 1994) and tissue culture techniques (e.g. colchicine treatment) are used to manipulate chromosome numbers (Harvey et al. 1995). Microsatellite and RAPD markers are used for cultivar identification (Cipriani et al. 1996; Fraser et al. 2001; Zhen et al. 2004) and RAPD markers are used to detect somaclonal mutation in micropropagated kiwifruit (Palombi and Damiano 2001). Sex-linked markers are used to screen seedling populations to remove males prior to planting in the field (Harvey et al. 1997; Gill et al. 1998). RAPD, microsatellite and AFLP markers have been developed (Huang et al. 1998; Fraser et al. 2004) for construction of *A. chinensis* linkage maps (Testolin et al. 2001; Fraser et al. 2004).

In New Zealand, field testing of transgenic kiwifruit is not likely to occur in the near future and genetically modified kiwifruit are not currently in development for market release. If transgenic kiwifruit are to be commercially developed it is most likely to occur in China or the United States where

consumer attitudes to genetically modified foods are more favorable. The functional testing of genes obtained from HortResearch's EST databases is focused on providing information for breeders to use in conjunction with the *A. chinensis* linkage map and characterizing enzymes for bioproduction of novel compounds.

**Acknowledgements.** We would like to thank E. Clegg and M. Napier for library assistance and all members of the kiwifruit genomics program team at HortResearch: A. Allen, L. Beuning, K. Bolitho, D. Cohen, R. Crowhurst, A. Gleave, K. Grafton, S. Green, R. Hellens, E. Hilario, K. Jamieson, B. Janssen, S. Karunairetnam, K. Klages, K. Marsh, R. Martinus, M. McNeillage, R. Newcomb, J. Nicholls, A. Richardson, G. Ross, R. Schaffer, R. Schröder, K. Snowden, M. Splawinski, K. Thodey, E. Walton, T. Wang, R.-M. Wu and Y. Yauk.

## References

- Atkinson RG, Gardner RC (1993) A polygalacturonase gene from kiwifruit (*Actinidia deliciosa*). *Plant Physiol* 103:669–670
- Atkinson RG, Cipriani G, Whittaker DJ, Gardner RC (1997) The allopolyploid origin of kiwifruit, *Actinidia deliciosa* (Actinidiaceae). *Plant Syst Evol* 205:111–124
- Baker EN (1980) Structure of actinidin, after refinement at 1.7 Ångstrom resolution. *J Mol Biol* 141:441–484
- Basile A, Ferrara L, Sepe J, De Sole P, Miranda R, Moscatiello V, Ielpo MTL, Simeone I, Sorbo S, Ricciardi L, Vuotto ML (2000) *Actinidia chinensis* leaf extracts in contact with Eubacteria and Eukarya. Proc 11th Int Symp on Bioluminescence and Chemiluminescence, Asilomar, California
- Belrose, Inc. (2004) Production and trade in fresh kiwifruit. World Kiwifruit Review 2004 Edition. Belrose, Inc., Pullman, Washington, pp 13–38
- Biasi R, Gutierrez P, Muganu M, Magro P, Bernabei M, Rugini E (2003) Modifications of growth pattern in kiwifruit and cherry induced by the T-DNA genes of *Agrobacterium rhizogenes*. Proc 47th Societa Italiana di Genetica Agraria (SIGA) Annual Congr, Verona, Abstract 4.11
- Chat J, Jauregui B, Petit RJ, Nadot S (2004) Reticulate evolution in kiwifruit (*Actinidia*, Actinidiaceae) identified by comparing their maternal and paternal phylogenies. *Am J Bot* 91:736–747
- Chen KS, Li F, Zhang SL (1999) The expression pattern of xyloglucan endotransglycosylase gene in fruit ripening of *Actinidia chinensis*. *Acta Bot Sin* 41:1231–1234
- Cheng CH, Seal AG, Boldingh HL, Marsh KB, MacRae EA, Murphy SJ, Ferguson AR (2004) Inheritance of taste characters and fruit size and number in a diploid *Actinidia chinensis* (kiwifruit) population. *Euphytica* 138:185–195
- Cipriani G, Di Bella R, Testolin R (1996) Screening RAPD primers for molecular taxonomy and cultivar fingerprinting in the genus *Actinidia*. *Euphytica* 90:169–174
- Cipriani G, Testolin R, Gardner R (1998) Restriction-site variation of PCR-amplified chloroplast DNA regions and its implication for the evolution and taxonomy of *Actinidia*. *Theor Appl Genet* 96:389–396
- Coimbra S, Torrão L, Abreu I (2004) Programmed cell death induces male sterility in *Actinidia deliciosa* female flowers. *Plant Physiol Biochem* 42:537–541
- Collins AR, Harrington V, Drew J, Melvin R (2003) Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* 24:511–515
- Crowhurst RN, Lints R, Atkinson RG, Gardner RC (1990) Restriction fragment length polymorphisms in the genus *Actinidia* (Actinidiaceae). *Plant Syst Evol* 172:193–203
- Duttaroy AK, Jorgensen A (2004) Effects of kiwi fruit consumption on platelet aggregation and plasma lipids in healthy human volunteers. *Platelets* 15:287–292

- Famiani F, Ferradini N, Standardi A, Hoza D, Stanica F (1997) In vitro regeneration of different *Actinidia* species. *Acta Hort* 444:133–138
- Ferguson AR (1990) Kiwifruit (*Actinidia*). *Acta Hort* 290:603–653
- Ferguson AR (1999) New temperate fruits: *Actinidia chinensis* and *Actinidia deliciosa*. In: Janick J (ed) Perspectives on new crops and new uses. ASHS Press, Alexandria, pp 342–347
- Ferguson AR, Ferguson LR (2003) Are kiwifruit really good for you? *Acta Hort* 610:131–138
- Ferguson AR, O'Brien IEW, Yan GJ (1997) Ploidy in *Actinidia*. *Acta Hort* 444:67–71
- Ferguson AR, Seal AG, McNeilage MA, Fraser LG, Harvey CF, Beatson RA (1996) Kiwifruit. In: Janick J, Moore JN (eds) Fruit breeding, vol II. Vine and small fruits crops. John Wiley, New York, pp 371–417
- Firsov AP, Dolgov SV (1997) Agrobacterial transformation of *Actinidia kolomicita*. *Acta Hort* 447:323–328
- Fraser LG, Kent J, Harvey CF (1995) Transformation studies of *Actinidia chinensis* Planch. *N Z J Crop Hortic Sci* 23:407–413
- Fraser LG, Harvey CF, Gill GP (2001) Application of microsatellite-based DNA profiling in *Actinidia* species. *Acta Hort* 546:401–405
- Fraser LG, Harvey CF, Crowhurst RN, De Silva HN (2004) EST-derived microsatellites from *Actinidia* species and their potential for mapping. *Theor Appl Genet* 108:1010–1016
- Fung RWM (2003) Characterisation of the multigene family of sucrose phosphate synthase in kiwifruit. PhD Thesis, University of Auckland, Auckland
- Fung RWM, Janssen B-J, Morris BA, Gardner RC (1998) Inheritance and expression of transgenes in kiwifruit. *N Z J Crop Hortic Sci* 26:169–179
- Fung RWM, Langenkämper G, Gardner RC, MacRae E (2003) Differential expression within an SPS gene family. *Plant Sci* 164:459–470
- Gavrovic-Jankulovic M, Cirkovic T, Vuckovic O, Atanaskovic-Markovic M, Petersen A, Gojic G, Burazer L, Jankov RM (2002) Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J Allergy Clin Immunol* 110:805–810
- Gilbert JM, Young H, Ball RD, Murray SH (1996) Volatile flavor compounds affecting consumer acceptability of kiwifruit. *J Sens Stud* 11:247–259
- Gill GP, Harvey CF, Gardner RC, Fraser LG (1998) Development of sex-linked PCR markers for gender identification in *Actinidia*. *Theor Appl Genet* 97:439–445
- Green S, Friel EN, Beuning L, Matich A, MacRae E (2004) A multifunctional germacrene D synthase. Patent no. WO04058814A1
- Harvey CF, Fraser LG, Kent J, Steinhagen S, McNeilage MA, Yan G-J (1995) Analysis of plants obtained by embryo rescue from an interspecific *Actinidia* cross. *Sci Hort* 60:199–212
- Harvey CF, Gill GP, Fraser LG, McNeilage MA (1997) Sex determination in *Actinidia*. 1. Sex-linked markers and progeny sex ratio in diploid *A. chinensis*. *Sex Plant Reprod* 10:149–154
- Hassall AK, Pringle GJ, MacRae EA (1998) Development, maturation, and postharvest responses of *Actinidia arguta* (Sieb. et Zucc.) Planch. *Ex Miq. fruit. N Z J Crop Hortic Sci* 26:95–108
- Hilario E, Bennell T, Crowhurst R, Fraser L, McNeilage M, Rikkerink E, MacRae E (2004) Construction of kiwifruit sequence-ready BAC contig maps by overgo hybridisation and their use for the targeted region around the sex locus. *Proc Int Conf on Bioinformatics*, Auckland, p 83
- Hirsch AM, Testolin R, Brown S, Chat J, Fortune D, Bureau JM, De Nay D (2001) Embryo rescue from interspecific crosses in the genus *Actinidia* (kiwifruit). *Plant Cell Rep* 20:508–516
- HortResearch (2005) HortResearch Kiwifruit Genomics Programme. HortResearch, Auckland
- Hou F, Sun Y, Chen F, Li X (1995) Immunopharmacological effects of polysaccharide from the stem of *Actinidia arguta* (Sieb. et Zucc.) *Ex Miq. Zhongguo Zhong Yao Za Zhi* 20:42–44
- Huang W-G, Cipriani G, Morgante M, Testolin R (1998) Microsatellite DNA in *Actinidia chinensis*: isolation, characterisation, and homology in related species. *Theor Appl Genet* 97:1269–1278
- Jaeger SR, Rossiter KL, Wismer WV, Harker FR (2002) Consumer-driven product development in the kiwifruit industry. *Food Qual Prefer* 14:187–198
- Janssen B-J, Gardner RC (1993) The use of transient GUS expression to develop an *Agrobacterium*-mediated gene-transfer system for kiwifruit. *Plant Cell Rep* 13:28–31

- Jordan MJ, Margaria CA, Shaw PE, Goodner KL (2002) Aroma active components in aqueous kiwi fruit essence and kiwi fruit puree by GC-MS and multidimensional GC/GC-O. *J Agric Food Chem* 50:5386–5390
- Keeling J, Maxwell P, Gardner RC (1990) Nucleotide sequence of the promoter region from kiwifruit actinidin genes. *Plant Mol Biol* 15:787–788
- Kim YK, Kang HJ, Lee KT, Choi JG, Chung SH (2003) Anti-inflammation activity of *Actinidia polygama*. *Arch Pharm Res* 26:1061–1066
- Kobayashi S, Nakamura Y, Kaneyoshi J, Higo H, Higo K (1996) Transformation of kiwifruit (*Actinidia chinensis*) and trifoliate orange (*Poncirus trifoliata*) with a synthetic gene encoding the human epidermal growth factor (hEGF). *J Jpn Soc Hortic Sci* 64:763–769
- Kobayashi S, Ding CK, Nakamura Y, Nakajima I, Matsumoto R (2000) Kiwifruits (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside). *Plant Cell Rep* 19:904–910
- Kusaba S, Kano-Murakami Y, Matsuoka M, Matsuta N, Sakamoto T, Fukumoto M (1999) Expression of the rice homeobox gene, *OSH1*, causes morphological changes in transgenic kiwifruit. *J Jpn Soc Hortic Sci* 68:482–486
- Laing WA, Bulley S, Wright M, Cooney J, Jensen D, Barraclough D, MacRae E (2004a) A highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. *Proc Natl Acad Sci USA* 101:16976–16981
- Laing WA, Frearson N, Bulley S, MacRae E (2004b) Kiwifruit L-galactose dehydrogenase: molecular, biochemical and physiological aspects of the enzyme. *Funct Plant Biol* 31:1015–1025
- Langenkämper G, McHale R, Gardner RC, MacRae E (1998) Sucrose-phosphate synthase steady-state mRNA increases in ripening kiwifruit. *Plant Mol Biol* 36:857–869
- Langenkämper G, Fung RWM, Newcomb RD, Atkinson RG, Gardner RC, MacRae EA (2002) Sucrose phosphate synthase genes in plants belong to three different families. *J Mol Evol* 54:322–332
- Ledger SE, Gardner RC (1994) Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var. *deliciosa*). *Plant Mol Biol* 25:877–886
- Lee H, Lin JY (1988) Antimutagenic activity of extracts from anticancer drugs in Chinese medicine. *Mutation Res* 204:229–234
- Li Z, Huang H, Jiang Z, Li J, Kubisiak TL (2003) Phylogenetic relationships in *Actinidia* as revealed by RAPDs and PCR-RFLPs of mtDNA. *Acta Hort* 610:387–396
- Lin E, Burns DJW, Gardner RC (1993) Fruit developmental regulation of the kiwifruit actinidin promoter is conserved in transgenic petunia plants. *Plant Mol Biol* 23:489–499
- Lucas JSA, Grimshaw KEC, Collins K, Warner JO, Hourihane JOB (2004) Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin Exp Allergy* 34:1115–1121
- MacDiarmid CWB, Gardner RC (1993) A cDNA sequence from kiwifruit homologous to 1-aminocyclopropane-1-carboxylic acid oxidase. *Plant Physiol* 101:691–692
- MacRae E, Atkinson R (2003) Multiple gene copies: carrying out biochemical and molecular studies in kiwifruit. *Acta Hort* 610:457–466
- MacRae E, Redgwell R (1992) Softening in kiwifruit. *Postharvest News Inf* 3:49N–52N
- MacRae EA, Quick WP, Benker C, Stitt M (1992) Carbohydrate metabolism during postharvest ripening in kiwifruit. *Planta* 188:314–323
- Marquis H, Bucheli P (1994) Inhibition of tomato pectin methylesterase by partially purified kiwi pectin methylesterase inhibitor protein. *Int J Food Sci Technol* 29:121–128
- Marsh K, Rossiter K, Lau K, Walker S, Gunson A, MacRae EA (2003) The use of fruit pulps to explore flavour in kiwifruit. *Acta Hort* 610:229–237
- Marsh K, Attanayake S, Walker S, Gunson A, Boldingh H, MacRae E (2004) Acidity and taste in kiwifruit. *Postharvest Biol Technol* 32:159–168
- Matich AJ, Young H, Allen JM, Wang MY, Fielder S, McNeilage MA, MacRae EA (2003) *Actinidia arguta*: volatile compounds in fruit and flowers. *Phytochemistry* 63:285–301

- Matsuta N, Iketani H, Hayashi T (1990) Effect of acetosyringone on kiwifruit transformation. *Jpn J Breed* 40:184–185
- Matsuta N, Iketani H, Hayashi T (1993) Transformation in grape and kiwifruit. In: Hayashi T, Omura M, Scott NS (eds) *Techniques on gene diagnosis and breeding in fruit trees*. Fruit Tree Research Station, Tsukuba, Ibaraki, pp 184–192
- McDonald B (1990) Precooling, storage, and transport of kiwifruit. In: Warrington IJ, Weston GC (eds) *Kiwifruit: science and management*. Ray Richards, Auckland, pp 429–459
- McGhie TK, Ainge GD (2002) Color in fruit of the genus *Actinidia*: carotenoid and chlorophyll compositions. *J Agric Food Chem* 50:117–121
- Moller M, Kayma M, Vieluf D, Paschke A, Steinhart H (1998) Determination and characterization of cross-reacting allergens in latex, avocado, banana, and kiwi fruit. *Allergy* 53:289–296
- Motohashi N, Shirataki Y, Kawase M, Tani S, Sakagami H, Satoh K, Kurihara T, Nakashima H, Mucsi I, Varga A, Molnár J (2002) Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. *J Ethnopharmacol* 81:357–364
- Nakamura Y, Sawada H, Kobayashi S, Nakajima I, Yoshikawa M (1999) Expression of soybean  $\beta$ -1,3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants. *Plant Cell Rep* 18:527–532
- Nayak AK, Beyl CA (1987) Callus induction and in vitro shoot multiplication of four species of *Actinidia*. *HortScience* 22:724
- Nishiyama I, Yamashita Y, Yamanaka M, Shimohashi A, Fukuda T, Oota T (2004) Varietal difference in vitamin C content in the fruit of kiwifruit and other *Actinidia* species. *J Agric Food Chem* 52:5472–5475
- Oliveira MM, Fraser LG (2005) *Actinidia* spp. Kiwifruit. In: Litz RE (ed) *Biotechnology of fruit and nut crops*. Biotechnology in Agriculture Series 29. CABI Publishing, Wallingford, Oxon, pp 2–27
- Oliveira MM, Barroso J, Pais MS (1991) Direct gene transfer into *Actinidia deliciosa* protoplasts: analysis of transient expression of the *CAT* gene using TLC autoradiography and a GC-MS based method. *Plant Mol Biol* 17:235–242
- Ollitrault-Sammarcelli F, Legave JM, Michaux-Ferriere N, Hirsch AM (1994) Use of flow cytometry for rapid determination of ploidy level in the genus *Actinidia*. *Sci Hort* 57:303–313
- Palombi MA, Damiano C (2001) Use of different molecular markers, RAPDs and SSRs, to investigate clonal stability in micropropagated kiwifruit (*Actinidia deliciosa* A. Chev.). *Acta Hort* 546:609–614
- Park YH, Chun EM, Bae MA, Seu YB, Song KS, Kim YH (2000) Induction of apoptotic cell death in human Jurkat T cells by a chlorophyll derivative (Cp-D) isolated from *Actinidia arguta* Planchon. *J Microbiol Biotechnol* 10:27–34
- Pastorello EA, Conti A, Pravettoni V, Farioli L, Rivolta F, Ansaloni R, Ispano M, Incorvaia C, Giuffrida MG, Ortolani C (1998) Identification of actinidin as the major allergen of kiwifruit. *J Allergy Clin Immunol* 101:531–537
- Paul W, Amiss J, Try R, Praekelt U, Scott R, Smith H (1995) Correct processing of the kiwifruit protease actinidin in transgenic tobacco requires the presence of the C-terminal propeptide. *Plant Physiol* 108:261–268
- Perera CO, Hallett IC (1991) Characteristics of the irritant (catch) factor in processed kiwifruit. *Acta Hort* 297:675–679
- Podivinsky E, Forster RLS, Gardner RC (1989) Nucleotide sequence of actinidin, a kiwi fruit protease. *Nucleic Acids Res* 17:8363
- Praekelt UM, McKee RA, Smith H (1988) Molecular analysis of actinidin, the cysteine proteinase of *Actinidia chinensis*. *Plant Mol Biol* 10:193–202
- Richardson AC, Marsh KB, Boldingh HL, Pickering AH, Bulley SM, Frearson NJ, Ferguson AR, Thornber SE, Bolitho KM, MacRae EA (2004) High growing temperatures reduce fruit carbohydrate and vitamin C in kiwifruit. *Plant Cell Environ* 27:423–435
- Rossiter KL, Young H, Walker SB, Miller M, Dawson DM (2000) The effects of sugars and acids on consumer acceptability of kiwifruit. *J Sens Stud* 15:241–250

- Rugini E, Pellegrineschi A, Mencuccini M, Mariotti D (1991) Increase of rooting ability in the woody species kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *Agrobacterium rhizogenes* *rol* genes. Plant Cell Rep 10:291–295
- Rugini E, Caricato G, Muganu M, Taratufolo C, Camilli M, Camilli C (1997) Genetic stability and agronomic evaluation of six-year-old transgenic kiwi plants for *rolABC* and *rolB* genes. Acta Hort 447:609–610
- Rush EC, Patel M, Plank LD, Ferguson LR (2002) Kiwifruit promotes laxation in the elderly. Asia Pac J Clin Nutr 11:164–168
- Sashida Y, Ogawa K, Mori N, Yamanouchi T (1992) Triterpenoids from the fruit galls of *Actinidia polygama*. Phytochemistry 31:2801–2804
- Sashida Y, Ogawa K, Yamanouchi T, Tanaka H, Shoyama Y, Nishioka I (1994) Triterpenoids from callus tissue of *Actinidia polygama*. Phytochemistry 35:377–380
- Schröder R, Atkinson RG, Langenkämper G, Redgwell RJ (1998) Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. Planta 204:242–251
- Seal AG (2003) The plant breeding challenges to making kiwifruit a worldwide mainstream fresh fruit. Acta Hort 610:75–80
- Simpson CG, MacRae E, Gardner RC (1995) Cloning of a polygalacturonase-inhibiting protein from kiwifruit (GenBank Z49063). Plant Physiol 108:1748
- Sugiyama S, Ohtsuki K, Sato K, Kawabata M (1996) Purification and characterization of six kiwifruit proteases isolated with two ion-exchange resins, Toyopearl-SuperQ and Bakerbond WP-PEI. Biosci Biotech Biochem 60:1994–2000
- Szankowski I, Briviba K, Fleschhut J, Schönherr J, Jacobsen H-J, Kiesecker H (2003) Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a PGIP gene from kiwi (*Actinidia deliciosa*). Plant Cell Rep 22:141–149
- Takano F, Tanaka T, Tsukamoto E, Yahagi N, Fushiya S (2003) Isolation of (+)-catechin and (–)-epicatechin from *Actinidia arguta* as bone marrow cell proliferation promoting compounds. Planta Med 69:321–326
- Testolin R, Huang WG, Lain O, Messina R, Vecchione A, Cipriani G (2001) A kiwifruit (*Actinidia* spp.) linkage map based on microsatellites and integrated with AFLP markers. Theor Appl Genet 103:30–36
- Testolin R, Messina R, Lain O, Cipriani G (2004) A natural sex mutant in kiwifruit (*Actinidia deliciosa*). N Z J Crop Hort Sci 32:179–183
- Uematsu C, Murase M, Ichikawa H, Imamura J (1991) *Agrobacterium*-mediated transformation and regeneration of kiwi fruit. Plant Cell Rep 10:286–290
- Voitenko V, Poulsen LK, Nielsen L, Norgaard A, Bindslev-Jensen C, Skov PS (1997) Allergenic properties of kiwi-fruit extract: cross-reactivity between kiwi-fruit and birch-pollen allergens. Allergy 52:136–143
- Walker S, Prescott J (2003) Psychophysical properties of mechanical oral irritation. J Sens Stud 18:325–346
- Walton EF, Podivinsky E, Wu R-M, Reynolds PHS, Young LW (1998) Regulation of proline biosynthesis in kiwifruit buds with and without hydrogen cyanamide treatment. Physiol Plant 102:171–178
- Walton EF, Podivinsky E, Wu R-M (2001) Bimodal patterns of floral gene expression over the two seasons that kiwifruit flowers develop. Physiol Plant 111:396–404
- Wang H, Ng TB (2002) Isolation of an antifungal thaumatin-like protein from kiwi fruits. Phytochemistry 61:1–6
- Wang T, Ran Y, Gleave A (2002) Factors enhancing *Agrobacterium*-mediated transformation of kiwifruit *Actinidia deliciosa*. Proc ComBio Combined Conf, Sydney, Abstract POS-WED-054
- Wang T, Ran Y, Atkinson RG, Gleave AP, Cohen D (2006) Transformation of *Actinidia eriantha*: A potential species for functional genomics studies in *Actinidia*. Plant Cell Rep 25: 425–431
- Wang Z-Y, MacRae EA, Wright MA, Bolitho KM, Ross GS, Atkinson RG (2000) Polygalacturonase gene expression in kiwifruit: relationship to fruit softening and ethylene production. Plant Mol Biol 42:317–328



- White A, de Silva HN, Requejo-Tapia C, Harker FR (2005) Evaluation of softening characteristics of fruit from 14 species of *Actinidia*. *Postharvest Biol Technol* 35:143–151
- Whittaker DJ, Smith GS, Gardner RC (1995) Three cDNAs encoding *S*-adenosyl-L-methionine synthetase from *Actinidia chinensis*. *Plant Physiol* 108:1307–1308
- Whittaker DJ, Smith GS, Gardner RC (1997) Expression of ethylene biosynthetic genes in *Actinidia chinensis* fruit. *Plant Mol Biol* 34:45–55
- Williams MH, Boyd LM, McNeilage MA, MacRae EA, Ferguson AR, Beatson RA, Martin PJ (2003) Development and commercialization of ‘Baby Kiwi’ (*Actinidia arguta* Planch.). *Acta Hort* 610:81–86
- Wurms K, Greenwood D, Sharrock K, Long P (1999) Thaumatin-like protein in kiwifruit. *J Sci Food Agric* 79:1448–1452
- Yamakawa Y, Chen LH (1996) *Agrobacterium rhizogenes*-mediated transformation of kiwifruit (*Actinidia deliciosa*) by direct formation of adventitious buds. *J Jpn Soc Hortic Sci* 64:741–747
- Yan G, Atkinson RG, Ferguson AR, McNeilage MA, Murray BG (1997) In situ hybridization in *Actinidia* using repeat DNA and genomic probes. *Theor Appl Genet* 94:507–513
- Yazawa M, Suginuma C, Ichikawa K, Kamada H, Akihama T (1995) Regeneration of transgenic plants from hairy root of kiwi fruit (*Actinidia deliciosa*) induced by *Agrobacterium rhizogenes*. *Breed Sci* 45:241–244
- Yoshizawa Y, Kawaii S, Urashima M, Fukase T, Sato T, Murofushi N, Nishimura H (2000a) Differentiation-inducing effects of small fruit juices on HL-60 leukemic cells. *J Agric Food Chem* 48:3177–3182
- Yoshizawa Y, Kawaii S, Urashima M, Fukase T, Sato T, Tanaka R, Murofushi N, Nishimura H (2000b) Antiproliferative effects of small fruit juices on several cancer cell lines. *Anticancer Res* 20:4285–4289
- Young H, Stec M, Paterson VJ, Mcmath K, Ball R (1995) Volatile compounds affecting kiwifruit flavor. *ACS Sym Ser* 596:59–67
- Zhen Y, Li Z, Huang H, Wang Y (2004) Molecular characterization of kiwifruit (*Actinidia*) cultivars and selections using SSR markers. *J Am Soc Hortic Sci* 129:374–382
- Zina AM, Bundino S (1983) Contact urticaria to *Actinidia chinensis*. *Contact Dermatitis* 9:85



## Section II Trees

## II.1 Walnuts

M.T. BRITTON, C.H. LESLIE, G.H. McGRANAHAN, and A.M. DANDEKAR<sup>1</sup>

### 1 Introduction

#### 1.1 Walnut Botany

Walnuts belong to the genus *Juglans*, which includes the four sections *Juglans* (English/Persian walnut), *Rhysocaryon* (black walnuts, native to the Americas), *Cardiocaryon* (Japanese, Manchurian and Chinese walnuts, including heartnuts) and *Trachycaryon* (the butternut of eastern North America) (Manning 1978). *Juglans* is comprised of a single dichogamous monoecious species, *Juglans regia* L. The trees contain both male (catkins) and female (pistillate) flowers that display protandry (male maturing earlier than female) and protogyny (female maturing earlier than male) (Forde and Griggs 1972). This dichogamy promotes outcrossing. Thus, walnut production is dependent upon wind pollination and bloom overlap. Successful pollination of the pistillate flower leads to the formation of walnuts distinguished by a dehiscent hull that separates from the shell at maturity (Manning 1978).

#### 1.2 *Juglans regia*

Native to central Asia, *J. regia* grows as a wild or semi-cultivated tree in a wide area from southeastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. Also referred to as the Persian walnut, it was prized by the Romans and was utilized in medieval Europe as an herbal medicine, particularly for brain and scalp ailments. *J. regia* has been cultivated for its nut crop for several thousand years and thus is the most horticulturally developed and widely cultivated of all the walnut species. It was probably introduced into European commerce and agriculture by the ancient Greeks. Its introduction into North America was more recent, where it has commonly been referred to as the English walnut to distinguish it from the American black walnut (Leslie and McGranahan 1998).

Grafting techniques, developed in France, allowed the first selection, development and propagation of cultivars. Many of these were introduced into

---

<sup>1</sup> Department of Plant Sciences, University of California, 1 Shields Ave, Davis, California 95616, USA, e-mail: amdandekar@ucdavis.edu

California in the late 1800s where, seedling orchards derived from introduced Spanish and Chinese seed sources had been established previously. In ensuing years, selections of superior seedling trees found in orchards derived from these combined sources were propagated to form the basis of the California walnut industry (McGranahan and Leslie 1990; Forde and McGranahan 1996; Ramos 1998).

Successful implementation of grafting allowed not only the development of improved cultivars, but also a choice of rootstock. In much of the world, *J. regia* seedlings are used as rootstock, but in California the native black walnut species, *J. hindsii*, has been widely preferred for its enhanced vigor, salt tolerance and disease resistance. In the early 1900s Luther Burbank first observed the superior vigor of *J. hindsii* × *J. regia* hybrids that he named 'Paradox' (Whitson et al. 1914; Howard 1945). Most California walnut orchards are currently grown on either seedling Paradox or seedling *J. hindsii* rootstocks. The development of clonal rootstocks has been impeded by the difficulty of rooting walnut cuttings.

### 1.2.1 Breeding and Genetics

The major breeding objectives are to increase yield, quality and range of harvest dates, while decreasing the amount of chemical input required to control pests and diseases. For a recent review of *J. regia* breeding and genetics, see Dandekar et al. (2005).

### 1.2.2 Rootstocks

Nearly all walnut production in the United States, and increasingly worldwide, is derived from grafted scion varieties on seedling rootstocks. This reflects the difficulty of rooting walnut cuttings, which precluded, until recently, substantial use of either rooted scions or improved clonal rootstocks. Employment of rootstocks is driven by the need to deal with soil, environment, disease and pest problems (McGranahan and Catlin 1987). In California, *J. hindsii* (Northern California black walnut) was preferred for much of the first half of the 20th century as it is a native species adapted to several soil-related problems, including resistance to crown and root rot caused by *Armillaria mellea* (oak root fungus) and tolerance to waterlogging and drought (Smith et al. 1912). Today, the most popular rootstocks are the interspecific hybrids, the first of which was 'Paradox', a hybrid between *J. hindsii* and *J. regia* described in 1893 by Burbank (Whitson et al. 1914; Howard 1945).

The major objective for walnut rootstock breeding is vigor, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to establish rapidly a full-sized bearing canopy. Other objectives include resistance to diseases and pests, most notably *Phytophthora*, nematodes and crown gall, as well as tolerance to cherry leafroll virus and tolerance of

soil-related problems, including waterlogging and salt accumulation. There is interest in controlling tree size, but not at the cost of vigor.

### 1.2.3 Scions

The ideal walnut cultivar would be relatively late leafing to escape the rains that spread walnut blight (*Xanthomonas campestris* pv. *juglandis*), precocious (yielding more than 500 kg ha<sup>-1</sup> in the fourth year) and vegetatively vigorous with bearing on both terminal and lateral shoots. It would have a low incidence of pistillate flower abscission and fruit drop and would not be alternate bearing. It would have high production capacity with low chemical input required. The harvest season would end in early October. The nutshell would be relatively smooth and well sealed and would make up no more than 50% of the nut weight. The nuts would fit the category of large or jumbo. The kernel would be plump and light colored, weighing about 7–8 g and be extracted easily in halves. The tree would be resistant to pests and diseases.

## 1.3 *Juglans nigra*

American black walnut (*J. nigra* L.) is a native hardwood species found throughout the eastern United States. Nearly all of this is in natural stands, in which less than 11% of the trees are walnut. Fewer than 14,000 acres are planted black walnut orchards (Shifley 2004). Although 2 million pounds per year of nutmeats are produced (Hammons et al. 2004), the trees also command high value for lumber and veneer. In the first half of the 20th century, the number of quality trees declined due to overharvesting of timber and clearing of land for agriculture. By the 1960s a program of tree planting had begun (Victory et al. 2004). Since then, this natural resource has been well managed. The number of trees, volume of lumber and quality has steadily increased over the last 40 years (Shifley 2004).

### 1.3.1 Breeding and Genetics

Breeding objectives include climatic adaptation, vigor and growth rate, form, fecundity and pest resistance. Because black walnut is grown for timber and veneer as well as nut production, trees that grow straight, with few lateral branches, and possess a large amount of heartwood with good color are very desirable (Woeste and McKenna 2004). Genetic improvement is accomplished primarily by testing of progeny derived from wild trees. By the early 1980s, walnut improvement programs had been established in 11 states in the US. However, it was found that significant interactions between environment and genotype greatly influenced the phenotypes of trees grown in the various climates, complicating the task of progeny evaluation (Victory et al. 2004).

Recent reviews of black walnut breeding have been presented by Tourjee and Gradziel (2001), Reid et al. (2004) and Woeste and McKenna (2004).

Walnut anthracnose, resulting from a fungal infection by *Gnomonia leptostyla*, is the only disease for which resistance is actively sought in breeding programs. Crosses were made between *J. nigra* and other walnut species including *J. regia*, *J. hindsii* and *J. ailantifolia* (Japanese walnut). Progeny testing indicated that *J. regia* and *J. ailantifolia* are potential sources of anthracnose resistance (Victory et al. 2004).

1.3.2 Propagation

Few viable seeds are available for propagation due to low production, predation and poor germination rates. Therefore, vegetative propagation is important to black walnut. Grafting has been employed, with a high proportion of successful grafts. More recently, tissue culture protocols have been developed (Victory et al. 2004).

2 Economic Importance

Worldwide, nearly 1.5 million metric tons of walnuts are produced annually. The top ten walnut producing and exporting countries and the value of the exports are listed in Tables 1 and 2. Within the United States, 100% of Persian walnut (*Juglans regia*) production is in California, where it is one of the top 20 agricultural commodities, with an annual production value of more than \$350 million. (California Department of Food and Agriculture 2004).

**Table 1.** Leading walnut producing countries in the world [FAOSTAT Agricultural Production data, Crops Primary, Walnuts (updated 20 December 2004), <http://faostat.fao.org/faostat/>]

Country	Rank	Production (Mt)	Percentage of world production	Area harvested (ha)
China	1	415,000	27.96	185,000
United States	2	294,840	19.86	86,000
Iran	3	160,000	10.78	60,000
Turkey	4	125,000	8.42	59,000
Ukraine	5	68,000	4.58	28,000
Romania	6	37,000	2.49	2,000
India	7	31,000	2.09	30,500
France	8	30,000	2.02	15,000
Egypt	9	27,000	1.82	5,000
Serbia and Montenegro	10	23,600	1.59	13,200
All others		296,412	18.38	117,930
Total		1,484,252	100.00	623,630

**Table 2.** Leading walnut exporting countries in the world [FAOSTAT Agricultural and Food Trade data, Crops and Livestock Primary and Processed. Total of Shelled and Unshelled Walnuts (updated 7 December 2004), <http://faostat.fao.org/faostat/>]

Country	Rank	Exports (Mt)	Exports (× \$1000)	Percentage of world exports
United States	1	83,253	215,674	41.06
Mexico	2	30,718	76,900	15.15
France	3	15,112	39,523	7.45
China	4	9,634	27,213	4.75
Moldova	5	9,564	23,070	4.72
Ukraine	6	9,297	18,864	4.59
Romania	7	8,913	19,809	4.40
Chile	8	7,182	22,908	3.54
India	9	6,301	21,330	3.11
Bulgaria	10	3,257	4,739	1.61
All Others		19,505	64,292	9.62
Total		202,736	534,322	100.00

### 3 Current Research and Development

#### 3.1 Tissue Culture

##### 3.1.1 Micropropagation

Walnuts have traditionally been propagated by grafting onto seedling rootstock. Micropropagation has been investigated for propagation of cultivars on their own roots, for production of selected rootstock clones, and for development of genetically engineered plants. Commercially, walnuts are micropropagated in only one laboratory in Spain (López 2001).

The first reports of micropropagation of Persian walnut are from the early 1980s (Chalupa 1981; Rodriguez 1982a, b; Somers et al. 1982; Caruso 1983; Cossio and Minolta 1983; Driver and Kuniyuki 1984). These techniques have been reviewed (McGranahan et al. 1987; Preece et al. 1989; Leslie and McGranahan 1992). DKW medium (Driver and Kuniyuki 1984) was developed specifically for walnut, but success has also been obtained on MS medium (Murashige and Skoog 1962). A comparison of different media for *J. regia* conducted by Saadat and Hennerty (2002) found that DKW was optimal when  $2.2 \text{ g l}^{-1}$  Phytagel was used as the gelling agent.

Walnuts are initiated into culture by introducing disinfested nodal segments of vigorous field- or glasshouse-grown shoots. Multiplication occurs through axillary shoot proliferation. Rapid transfer (two to five times per week) is essential after introduction into culture until discoloration of the medium is

no longer evident. Once established, cultures need relatively frequent transfer (two times per month) for optimum growth. It has been shown recently that the addition of 1 mM phloroglucinol to the multiplication media increases subsequent rooting (Leslie et al. 2006).

Techniques for rooting are still under investigation and rooting ability is clone specific. The most promising rooting technique utilizes a two-phase system originally developed by Jay-Allemand et al. (1992) and subsequently modified by Navatel and Bourrain (2001) and Vahdati et al. (2004). Roots are induced by placing shoots on MS medium containing auxin and at least 40 g l<sup>-1</sup> sucrose for 6–8 days in the dark. Induced shoots are then transferred to a root development medium consisting of a mix of one-quarter-strength basal DKW medium and vermiculite (to improve aeration) and maintained in the light for 3–4 weeks until roots are visible. An alternative method is to treat unrooted microshoots with auxin, and root them in vermiculite in a fog chamber (Leslie et al. 2006). This technique results in a lower percentage of rooted shoots. However, those that were rooted using this procedure had very little callus and produced roots that were more fibrous than microshoots rooted in vitro.

Rooted shoots are planted in a well-drained potting soil and are acclimated by growth in a fog chamber for 2 weeks, followed by a week or two on a shaded glasshouse bench. However, the stress of acclimatization can lead to arrest of the apical meristem. Budbreak can be improved by application of 25 ml l<sup>-1</sup> Promalin [a commercial product containing 1.8% gibberellic acid (a mixture of GA4 and GA7) and 1.8% benzylaminopurine (BAP, Valent Biosciences, Walnut Creek, California)] as a foliar spray to stimulate growth (Vahdati et al. 2004).

In a study comparing variations of this protocol on several different walnut cultivars, a correlation was found between the vigor of adult trees and their rooting ability. Although smaller microshoots can be produced more rapidly and more efficiently in vitro, longer microshoots appear to acclimatize better in the glasshouse, perhaps due to a greater internal reservoir of carbohydrates and a more lignified stem, which may lead to increased pathogen resistance (Vahdati et al. 2004).

Bisbis et al. (2003) also found a link between shoot lignification and root development, concluding that signals from the roots, as well as auxin, enhance lignin formation. This lignin was only found in xylem cells, and was correlated positively with the number of developing roots, beginning immediately after treatment with exogenous auxin (Kevers et al. 2004). This appears to trigger peroxidases that are involved in the process of building cell walls. Peroxidase activity had previously been shown to be a good marker for walnut rooting (Gaspar et al. 1992; Ripetti et al. 1994).

While the above protocol is generally effective, it is often found that media must be optimized for specific cultivars and clones. Dolcet-Sanjuan et al. (2004) investigated the influence of different factors on root formation of micropropagated walnut shoots from several genotypes, including *J. regia* and *J. nigra* × *J. regia* hybrids, and concluded that the ability to acclimatize was dependent both on genotype and the juvenility of the starting plant material. Microshoots



derived from embryos rooted much more easily than those originally started from adult plant material.

Shoots of black walnut have also been micropropagated using both liquid DKW (Pearson et al. 2001) and Long and Preece media (Pearson et al. 2000). It is important to note that the media composition generally must be optimized for each walnut species and cultivar for successful tissue culture.

### 3.1.2 *Micrografting to Eliminate Viruses*

No information is available regarding the elimination of viruses via micrografting in walnut.

### 3.1.3 *Somatic Cell Genetics*

Very little work has been carried out on the somatic cell genetics of walnuts. Intact protoplasts have been isolated from soft stem tissue of micropropagated shoots (McGranahan, unpublished data), but no additional work has been performed to induce the protoplasts to synthesize new cell walls or to utilize the protoplast-derived cells.

A tetraploid walnut cultivar, 'Mitsuru', derived by colchicine treatments, has been recently compared to the diploid 2X Mitsuru and analyzed for pollen characteristics (size, germination rate, fertility, etc.) (Yajima et al. 2003). The 2X Mitsuru is known as a Shinano cultivar and was derived from a cross between *J. regia* var. *orientis* Kitamura (Teuchi walnut) and the Persian walnut (*J. regia* L.). Shoots of Mitsuru were exposed to a 0.4% colchicine solution containing 1 ppm of NAA ( $\alpha$ -naphthaleneacetic acid) to produce the tetraploid 4X Mitsuru walnuts. These were confirmed by chromosome analysis of shoot tips of the seedlings obtained from open- and self-pollinated plants (Yajima et al. 1997).

### 3.1.4 *Somatic Embryogenesis*

Development of embryos from asexual tissues has been a very useful tool in genetic improvement, particularly because tissues (i.e. leaf discs, protoplasts) commonly used in other plants have not been regenerated to plants in walnut. Somatic embryogenesis has been used to generate triploids (Tulecke and McGranahan 1988), intergeneric hybrids (McGranahan et al. 1986) and genetically transformed clones (McGranahan et al. 1988, 1990; Dandekar et al. 1989).

The techniques were developed for *J. regia* (Tulecke and McGranahan 1985) but have been applied to other species (Neuman et al. 1993). Immature cotyledonary explants harvested from developing nuts, cultured on conditioning medium for 2–4 weeks and then placed on basal DKW medium, will develop small white somatic embryos from single cells (Polito et al. 1989) on the explants after 8–16 weeks. These new embryos are repetitively embryogenic and, with monthly subculturing, cultures can be maintained for years.

For initiation and multiplication, embryos are maintained at room temperature in the dark. In the light, embryos turn green and a certain percentage will germinate. Germination frequency can be increased following desiccation over a saturated salt solution ( $\text{Zn}_2\text{SO}_4$ ,  $\text{NH}_4\text{NH}_3$ ,  $\text{MgCl}_2$ ) until the embryos are white, with the consistency of popcorn, but not until they have turned brown. Embryos are then returned to DKW basal medium to germinate. Additional details of methods and progress in walnut embryogenesis are given in the reviews by Preece et al. (1995) and Tulecke et al. (1995).

A major challenge today in walnut tissue culture is to obtain embryogenesis or organogenesis from maternal tissue. This is important because embryos from zygotic tissue do not allow the exact genotype to be predicted, even if both parents are known. Therefore, the use of these embryos for genetic transformation can result in plants that can be brought into the breeding program in order to combine desirable traits. Efforts to generate somatic embryos from nucellus have been unsuccessful (Aly et al. 1992). Repetitively embryogenic cultures have been obtained from immature anther tissue, but only from the cultivar Chandler. Recently, a modified protocol allowed a somatic embryo line to be generated from immature anthers of a Paradox hybrid (McGranahan, unpublished data). This may provide a source of elite rootstock tissue for genetic transformation. The protocol consists of three stages. First, dissected anthers are placed in liquid flask culture in the dark for 7–8 weeks to induce callus production. Second, callus from the flask is transferred to semi-solid DKW medium with hormones for 2 weeks. Third, the callus is transferred to semi-solid DKW medium lacking hormones and subcultured at 2-week intervals.

### 3.1.5 Triploid Recovery from Endosperm

Walnut endosperm has been used to generate triploids ( $3n = 48$ ) through somatic embryogenesis (Tulecke et al. 1988). Endosperm was cultured 4–12 weeks post-pollination using standard techniques for somatic embryogenesis. The cultivars Payne, Early Ehrhardt and Manregian produced repetitively embryogenic cultures. Triploids from endosperm of Manregian seed are maintained in the *Juglans* germplasm collection at the University of California, Davis. Trees flower and set nuts but embryos do not develop, so the shells are empty and very small.

### 3.1.6 Cryopreservation

Zygotic embryos, somatic embryos and pollen have been successfully stored under liquid nitrogen (LN). Walnut pollen with less than 7.5% moisture content survives cryostorage for at least 1 year (Luza and Polito 1988). Satisfactory moisture status is obtained by air-drying pollen for 24 h after anthesis. Zygotic embryo axes of *J. regia* survive LN storage after desiccation to 5–10% moisture content (Pence 1990). Somatic embryos survive when treated with 0.2 M

sucrose for 24 h and then desiccated to 30–40% moisture before LN storage (Setka 1994).

### 3.2 Transgenic Technology

Tree crops, such as walnut, are highly heterozygous and have very long generation times, which makes traditional breeding difficult. A large investment in both time and land is needed to grow seedlings to maturity in order to determine nut quality. It is therefore advantageous to be able to introduce specific traits into existing elite cultivars. These traits may come from within the germplasm of the genus or from other organisms. Desirable traits for walnut include disease and pest resistance, as well as good nut and timber quality. Herbicide tolerance would allow weeds to be controlled economically in nurseries and young orchards without damage to the trees.

#### 3.2.1 Genetic Transformation

Genetic transformation is the process of asexually introducing DNA into plants, typically via *Agrobacterium tumefaciens*. Walnuts are susceptible to *A. tumefaciens* and were one of the first woody plants to be transformed and express foreign genes (Dandekar et al. 1988; McGranahan et al. 1988). Use of transgenics can allow existing elite cultivars to be improved without the long generation times required for traditional breeding. Transformation of rootstocks such as Paradox may allow desirable traits to be incorporated without changing the genetic makeup of the scion or nuts and without the potential for horizontal gene transfer.

#### 3.2.2 Objectives

Plant transformation is useful for recalcitrant problems in walnut improvement, including resistance to diseases and pests. Codling moth is the key insect pest of walnut and chemical application is the main method of controlling this insect, because there is little genetic resistance in walnut germplasm that can be utilized. Crown gall is a serious problem for many fruit, nut and ornamental crops, greatly diminishing productivity. Walnuts are very susceptible to this disease. Losses are incurred from both contaminated nursery stock and infected orchard trees. Current prophylactic measures and mechanical removal of galls have not adequately controlled the problem.

#### 3.2.3 Protocol

Walnut is transformed by inserting genes into embryogenic cultures, since *A. tumefaciens* readily infects young proliferating somatic embryos (McGrana-

han et al. 1988). Since new embryos develop from single epidermal cells (Polito et al. 1989), transformed cells produce entirely transformed embryos and chimeras are eliminated. Several independent transgenic lines can be obtained from a single embryo, indicating multiple infection sites on the surface of the walnut embryo (McGranahan et al. 1990). This feature makes the walnut transformation system very efficient. A detailed protocol for the transformation of walnut has been published (Dandekar et al. 1989; Leslie et al. 2006). The efficiency of selecting transformed embryos can be improved by the introduction of a scorable marker along with the desired transgene (McGranahan et al. 1990; Escobar et al. 2000).

Transgenic embryos are subsequently germinated to produce uniformly transformed plants. In 1989, this method was used to produce walnut trees that were the first transformed woody fruit or nut tree to be field tested. These trees bore nuts and the introduced genes were found to be both stably incorporated and inherited in a simple Mendelian fashion (McGranahan, unpublished data).

Transformation of black walnut (*J. nigra* L.) has also been reported recently (Bosela et al. 2004) through the use of somatic embryos. For black walnut the most desirable traits include herbicide resistance, control of flowering and the development of heartwood.

### 3.2.4 Regeneration

Most of the success in regenerating walnut from tissue explants has come from embryogenesis and not organogenesis. Walnuts seem to be highly recalcitrant to undergoing organogenesis and form shoots from callus or tissue explants. This has posed a problem in the use of transgenics to improve existing walnut cultivars and particularly rootstocks, since the elite rootstocks are interspecific hybrids and exhibit moderate to severe sterility. Research is currently ongoing to develop novel regeneration technologies, including the use of genes identified in *Arabidopsis* whose over-expression results in a high frequency of embryo formation from a wide range of tissues (Dandekar, unpublished data).

## 3.3 Molecular Genetics

Molecular genetics encompasses a broad suite of technologies for analysis of genes and their expressed products. Unfortunately, research at the molecular level in many horticultural tree crops has lagged, in large part due to the time and effort it takes to generate data. This is now changing, as new and more profound methods are available to investigate the 'gene space' of crops such as walnut. Genomic approaches that involve a non-biased data collection of genetic information are now available to the scientific community (Bent 2000; Weinstein 2002). Robotics is simplifying the analysis of thousands of genes, with the genetic data being analyzed by specific computer programs

and the useful data stored in public databases. These tools will dramatically improve the availability of genetic information for crops like walnut in the near future.

### 3.3.1 Gene Cloning

Gene cloning is an important endeavor because tree crops are likely to possess many unique genes that may not be discovered in other plants. However, little progress has been reported primarily due to the few walnut researchers worldwide and the availability of resources to fund these endeavors. One of the useful sources of information on cloned walnut genes is GenBank (NCBI), the public repository for DNA sequences. Currently, this database has over 5000 entries. Table 3 lists the number of GenBank entries by walnut species.

Genes specific to walnut currently being studied include those involved in tannin, naphthoquinone, unsaturated fatty acid and flavonoid biosynthesis. Several of the genes involved in the biosynthetic pathway of flavonoids have been identified (Beritogoli et al. 2002). Recently, homologues of two Arabidop-

**Table 3.** Walnut DNA sequence entries in GenBank (October 2006)

Species	Chloroplast genes	Ribosomal genes	Mitochondrial genes	Micro-satellites	Nuclear encoded genes	Total entries
<i>J. ailantifolia</i>	6	2	0	0	0	8
<i>J. australis</i>	6	2	0	0	0	8
<i>J. boliviana</i>	1	1	0	0	0	2
<i>J. californica</i>	25	5	0	0	0	30
<i>J. cathayensis</i>	7	3	0	0	0	10
<i>J. cinerea</i>	7	3	0	0	0	10
<i>J. guatemalensis</i>	5	2	0	0	0	7
<i>J. hindsii</i>	34	6	0	0	0	40
<i>J. hopeiensis</i>	4	1	0	0	0	5
<i>J. major</i>	19	7	0	0	0	26
<i>J. mandshurica</i>	16	3	1	0	0	20
<i>J. microcarpa</i>	22	7	0	0	0	29
<i>J. mollis</i>	4	1	0	0	0	5
<i>J. neotropica</i>	7	1	0	0	0	8
<i>J. nigra</i>	26	9	1	39	10	85
<i>J. nigra</i> × <i>J. regia</i>	0	0	0	0	12	12
<i>J. olanchana</i>	5	2	0	0	0	7
<i>J. regia</i>	14	5	0	0	5040	5059
<i>J. sigillata</i>	5	1	0	0	0	6
<i>J. sp.</i> NSW 476481	1	0	0	0	0	1
Total	214	61	2	39	5062	5378

sis genes controlling floral transition and flower differentiation were cloned from microshoots of Early Mature (EM) walnuts (Breton et al. 2004). EM walnut trees can flower within 1 year of germination and develop into trees that are smaller, bushier and more cold-hardy than other *J. regia* genotypes. These traits may be useful in transgenic plants and traditional breeding programs.

A key determinate of walnut kernel quality is the oil content, about 90% of which is polyunsaturated. Of that, omega 3-fatty acid  $\alpha$ -linolenic acid (ALA) comprises 25% (Greve et al. 1992). The presence of unsaturated fatty acids is an important factor in the rancidification of walnuts, in which these acids are oxidized, thus reducing the shelf life of walnut kernels (Greve et al. 1992). The omega-3 fatty acids have been shown to play an important role in growth and development, nutrition and disease prevention. Nutritional studies have demonstrated that walnut consumption can reduce the incidence of coronary heart disease. The genes encoding the various fatty acid desaturases involved in the synthesis of polyunsaturated fatty acids, including *fad 2* and *fad 3*, have been cloned from walnut (Dandekar, unpublished data).

### 3.3.2 Marker Assisted Selection

The selection of desired progeny in a breeding program can be facilitated by the use of molecular markers. Cloned genes can be excellent markers as long as they display some polymorphism. Additionally, molecular markers can be used in more traditional genetic strategies utilizing linkage mapping and map-based cloning. Molecular markers have improved the efficiency of linkage mapping, allowing identification of discrete DNA segments where genes of interest reside (Camilleri et al. 1998). Some mapping efforts are ongoing in black (Woeste et al. 2002) and Persian (Aradhya et al. 2001) walnuts. These mapping efforts utilize AFLP and microsatellite markers.

Microsatellite loci are being used to fingerprint walnut cultivars and, most recently, inter-simple sequence repeat markers (ISSR) have been used to determine the genetic relationships of closely related walnut cultivars (Potter et al. 2002a; Orel et al. 2003; Pollegioni et al. 2003, 2006; Dangl et al. 2005; Foroni et al. 2005). This is important to verify and document breeding programs, as well as to understand how pollen flows within and between orchards. Sequences from the intergenic regions of chloroplast and ribosomal genes have been used to investigate the phylogenetic relationships between *Juglans* species and cultivars (Potter et al. 2002b; Aradhya et al. 2006). Isozymes have also been used to determine genetic relationships between cultivars from different geographic locations (Ninot and Aleta 2003; Vyas et al. 2003). Marker assisted selection is currently in use to identify individuals resistant to cherry leaf roll virus among a backcross population of Persian  $\times$  black walnut (Woeste et al. 1996a, b).

Black walnut population genetics studies have been conducted over the last 25 years with allozyme markers to determine the amount of genetic diversity within the populations comprising the species (Victory et al. 2004).

More recently, microsatellites (SSRs) have been identified (Woeste et al. 2002; Robichaud et al. 2006).

### 3.3.3 Functional Genomics

Enzymes in the phenylpropanoid pathway from phenylalanine lead to the biosynthesis of a range of natural products, including flavonoids. Genes for these enzymes, including the key enzyme chalcone synthase, have been investigated in walnuts. Walnuts expressing antisense chalcone synthase were found to be deficient in the accumulation of flavonoids, but interestingly, these deficient plants showed an increase in adventitious root formation (El Euch et al. 1998). These results contrast with other root initiation studies using walnut cotyledons in which adventitious rooting was observed to occur when the appearance of the lateral root primordia coincided with the expression of chalcone synthase at the same location (Ermel et al. 2000). The genes in the phenylpropanoid and flavonoid pathways were also studied in order to understand the accumulation of flavanols during heartwood formation in black walnut (Beritogoli et al. 2002). The authors concluded that flavanol synthesis was due to the increased transcriptional activity of genes in the phenylpropanoid pathway in black walnut sapwood cells that are undergoing the transition to heartwood.

Naphthoquinone metabolism has also been investigated and proteins involved in some of the steps have been identified. Naphthoquinones are important for plant defense and may also be involved in developmental processes (Duroux et al. 1998).

Oil biosynthesis in the embryo is a major metabolic pathway and some effort has been directed at functional characterization of two key steps in the biosynthesis of polyunsaturated fatty acids. Transgenic walnut embryos expressing antisense *fad 2* or sense *fad 3* have been developed (Dandekar, unpublished data) and some of the lines show alterations in the profile and composition of fatty acids. It is hypothesized that expression of antisense *fad 2* will suppress the interconversion of oleic to linoleic acid, leading to an increase in the accumulation of the monosaturated oleic acid. The expression of sense *fad 3* is aimed at overexpressing the enzyme involved in the conversion of linoleic acid to the omega 3 fatty acid linolenic acid. These studies will be useful in developing walnut lines with high oleic acid (monounsaturated fatty acid) content for stability and also lines with increased omega 3 fatty acids.

## 4 Practical Applications of Transgenic Plants

A major accomplishment has been the development of walnut trees expressing resistance to codling moth. Various insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* were tested by incorporating the proteins into insect



diets (Vail et al. 1991). The cryIA(c) protein was found to be the most effective. However, transformation of walnut using the bacterial gene failed due to lack of expression resulting from the codon bias of the bacterial gene sequence (Dandekar et al. 1994). A synthetic gene corrected this problem and high levels of codling moth mortality were observed when larvae were fed transgenic embryos (Dandekar et al. 1998). Laboratory tests have confirmed that high expressing genotypes are lethal to the codling moth larvae, and field trials are in progress (Vail et al. 1991; Dandekar et al. 1992, 1994, 1998; Leslie et al. 2001).

Recently, a gene silencing strategy was developed to produce resistance to crown gall disease in *Arabidopsis* and tomato, demonstrating for the first time the use of gene silencing to generate resistance to a major bacterial disease (Escobar et al. 2001). This approach has subsequently been successfully applied to walnuts (Escobar et al. 2002). Transgenic walnuts were highly resistant to galling. Resistant genotypes display an absence of macroscopic and microscopic indications of tumorigenesis after infection with a wide range of *A. tumefaciens* strains, indicating a broad-spectrum durable resistance (Escobar et al. 2003). These plants also provide a unique resource for examining fundamental questions about *Agrobacterium* biology and post-transcriptional gene silencing (PTGS) (Escobar et al. 2003).

Success has also been achieved in modifying tree architecture using the *rolABC* genes from *Agrobacterium rhizogenes*. Transgenic walnut trees expressing these genes show leaf curling, a marked reduction in internode length, and altered root architecture, but no increase in rootability (Vahdati et al. 2002).

Walnut is a tree crop whose nuts can be contaminated with aflatoxins, carcinogenic and teratogenic chemical compounds synthesized by members of the fungal genus *Aspergillus*. Many countries that import walnuts have set total aflatoxin action threshold levels at 4 ppb, significantly below the US Food and Drug Administration recommendation of 20 ppb. Because fungal infections often follow damage due to feeding of insects such as codling moth larvae, an important objective of breeding and genetic engineering is to develop lines that demonstrate insect resistance (Campbell et al. 2003). As discussed above, the engineering of walnuts to produce the insecticidal cryIA(c) protein shows promise of reducing codling moth damage and subsequent fungal infection.

Recently, it was discovered that kernels of the 'Tulare' variety of Persian walnut are able to suppress the production of aflatoxin (Mahoney et al. 2003). Evidence points to the high concentration of gallic acid in the pellicle (seed coat) as the factor that inhibits aflatoxin generation. The gene encoding shikimate dehydrogenase (SDH), the enzyme responsible for gallic acid production, has been cloned. Somatic embryos of the cultivar 'Chandler' were transformed to overexpress the SDH gene, resulting in the production of a high concentration of gallic acid and increased inhibition of aflatoxin production (Muir, unpublished data).

## 5 Conclusions and Future Challenges

A combination of conventional, *in vitro* and molecular approaches has facilitated considerable progress in diverse aspects of walnut improvement. Traditional crossing has contributed to the development of scion cultivars with improved yield, quality, harvest timing and virus resistance and to rootstocks selected for vigor, virus tolerance and nematode resistance. Selection among these crosses has been made more efficient by the development of molecular markers for blackline resistance, cultivar identification and germplasm diversity. Genomic tools will have a profound impact on the genetics of crops like walnut as they will provide key genetic resources that will afford a greater precision in ongoing breeding efforts. Over the next decade, the genomes of many crops will be sequenced, including walnuts, providing a simple list of all of the walnut genes. Such information will greatly enable the development of novel diagnostics and therapeutic approaches, as well as make breeding more targeted.

Micropropagation and somatic embryogenesis techniques have enabled the development of improved rootstocks and the implementation of gene transformation. The latter has already been used to develop walnuts resistant to codling moth (the key insect pest) and crown gall disease (a widespread and commercially important rootstock problem). Disease and pest resistance are significant targets as they also reduce the use of pesticides and thus have a profound impact both on productivity and the environment. Progress to combat disease and pest problems in walnut will be aided by advances made in biotechnology applications in other plants. Functional genomics approaches will provide a much clearer understanding of the metabolic processes that make walnuts unique. Functional genomics will also give us a clearer understanding of walnut metabolism and physiology, presenting additional opportunities to develop disease and pest resistance and to improve wood structure and kernel quality traits in the future. Furthermore, it should be possible to maximize the relationship between walnut components and the potential health benefits for consumers of walnuts and walnut products.

### 5.1 Emerging Opportunities

In recent years, a large body of evidence has accumulated from studies that points to health benefits derived from incorporating walnuts into a healthy diet. Diseases that appear to be improved by the sufferer consuming walnuts include cardiovascular and coronary heart disease (Zambon et al. 2000; Morgan et al. 2002; Zibaenezhad et al. 2003; Ros et al. 2004; Reiter et al. 2005), type 2 diabetes (Fukuda et al. 2004; Gillen et al. 2005), Alzheimer's disease (Chauhan et al. 2004), liver cytotoxicity (An et al. 2005), tooth decay (Jagtap and Karkera 2000) and acne (Qadan et al. 2005). Studies on the effects of walnuts and coronary heart disease have been reviewed by Feldman (2002). There is also

evidence that compounds contained in walnut may be useful in reducing the toxicity of broad-spectrum chemotherapy drugs (Haque et al. 2003).

The beneficial aspects of walnuts are derived from both their unique fatty acid composition and numerous products of secondary metabolism, including high levels of fiber, folate, polyphenolic compounds, tannins and the amino acid arginine (Ros et al. 2004). Although walnuts are high in total fat content, increased consumption of walnuts does not result in gains to body weight, because the resulting diet has a low proportion of saturated fat (Zambon et al. 2000; Gillen et al. 2005). The potential health benefits have led to research into how walnuts can be incorporated into other foods to increase their consumption. Recently, the addition of walnuts as filler to frankfurters was investigated (Colmenero et al. 2005).

The consumption of many nut species, including walnuts, can initiate an allergic reaction in susceptible persons. The major food allergen genes have been cloned, but elimination of the allergen proteins by conventional breeding is unlikely to be accomplished (Comstock et al. 2004). Therefore, this drawback to widespread walnut consumption could be addressed by genetic engineering to silence the genes involved. Walnut somatic embryos can be used as a model system to test many of these ideas as they are easily transformable and represent the edible portion of the walnut. Transgenic walnut somatic embryos could be cultured in vitro in bioreactors to produce valuable health-related products.

Walnuts may also be useful in phytoremediation programs, particularly where contaminated land could be planted for timber production. Recently, *J. regia* plants grown in lead-contaminated soil demonstrated the ability to accumulate high concentrations of lead in the root tissues, with little translocation to above-ground parts (Marmioli et al. 2005). Genetic transformation could be used to enhance the range and capacity of walnut rootstocks.

Biotechnology can also directly benefit developing countries where walnuts are grown. For example, extraction of kernels results in a large mass of shells, which are used for combustion. Research is now showing that this biomass can be converted to pyrolytic oil, which can be further refined to produce transportation quality fuels of equal or higher quality to those derived from petroleum (Onay et al. 2004). Crushed walnut and almond shells can also be combined with activated carbon to produce high-efficiency and low-cost filters for drinking water (Ahmedna et al. 2004).

## References

- Ahmedna M, Marshall W, Hussein A, Rao R, Goktepe I (2004) The use of nutshell carbons in drinking water filters for removal of trace metals. *Water Res* 38:1062–1068
- Aly MAM, Fjellstrom RG, McGranahan GH, Parfitt DE (1992) Origin of walnut somatic embryos determined by RFLP and isozyme analysis. *HortScience* 27:61–63
- An R, Kim H, Tian Y, Kim Y (2005) Free radical scavenging and hepatoprotective constituents from the leaves of *Juglans sinensis*. *Arch Pharm Res* 28:529–533

- Aradhya MK, Potter D, Woeste K, Simon CJ (2001) An integrated high-density AFLP-SSR map of Persian walnut *Juglans regia* L. HortScience 36:536
- Aradhya MK, Potter D, Simon CJ (2006) Origin, evolution, and biogeography of *Juglans* L: a phylogenetic perspective. Acta Hort 705:215–220
- Bent R (2000) Genomic biology. Cell 100:169–183
- Beritogoli I, Magel E, Abdel-Latif A, Charpentier J-P, Jay-Allemand C, Breton C (2002) Expression of genes encoding chalcone synthase flavanone 3-hydroxylase and dihydroflavonol 4-reductase correlates with flavanol accumulation during heartwood formation in *Juglans nigra*. Tree Physiol 22:291–300
- Bisbis B, Kevers C, Crevecoeur M, Dommes J, Gaspar T (2003) Restart of lignification in micro-propagated walnut shoots coincides with rooting induction. Biol Plant 47:1–5
- Bosela MJ, Smagh GS, Michler CH (2004) Genetic transformation of black walnut (*Juglans nigra*). In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) Black walnut in a new century. Proc 6th Walnut Council Research Symp, Lafayette, Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 45–58
- Breton C, Cornu D, Chriqui D, Sauvanet A, Capelli P, Germain E, Jay-Allemand C (2004) Somatic embryogenesis, micropropagation and plant regeneration of “Early Mature” walnut trees (*Juglans regia*) that flower in vitro. Tree Physiol 4:425–435
- California Department of Food and Agriculture (2004) California Agricultural Statistics 2003. California Agricultural Statistics Service, Sacramento, California, p 2
- Camilleri C, Laffleuril J, Macadre C, Varoquaux F, Parmentier Y, Picard G, Caboche M, Bouchez D (1998) A YAC contig map of *Arabidopsis thaliana* chromosome 3. Plant J 14:633–642
- Campbell B, Molyneux R, Schatzki T (2003) Current research on reducing pre- and post-harvest aflatoxin contamination of U.S. almond, pistachio, and walnut. J Toxicol Toxin Rev 22:225–266
- Caruso JL (1983) In vitro axillary shoot formation and rooting in black walnut mature embryos. In: Guries RP (ed) Proc 3rd North Central Tree Improvement Conf, Wooster, Ohio. North Central Tree Improvement Association, Madison, Wisconsin, pp 144–149
- Chalupa V (1981) Clonal propagation of broad-leaved forest trees in vitro. Comm Inst For Cech 12:255–271
- Chauhan N, Wang N, Wegiel J, Malik M (2004) Walnut extract inhibits the fibrillization of amyloid beta-protein, and also defibrilizes its preformed fibrils. Curr Alzheimer Res 1:183–188
- Colmenero FJ, Ayo MJ, Carballo J (2005) Physicochemical properties of low sodium frankfurter with added walnut: effect of transglutaminase combined with caseinate, KCl and dietary fibre as salt replacers. Meat Sci 69:781–788
- Comstock SS, McGranahan GH, Peterson WR, Teuber SS (2004) Extensive in vitro cross-reactivity to seed storage proteins is present among walnut (*Juglans*) cultivars and species. Clin Exp Allergy 34:1583–1590
- Cossio F, Minolta G (1983) Prove preliminary di coltura in vitro di embrioni isolati di noce (*Juglans regia* L) e confronto tra differenti combinazioni di Sali minerali. Riv Ortoflorofruttic Ital 67:287–298
- Dandekar AM, Martin LA, McGranahan GH (1988) Genetic transformation and foreign gene expression in walnut tissue. J Am Soc Hortic Sci 113:945–949
- Dandekar AM, McGranahan GH, Leslie CA, Uratsu SL (1989) *Agrobacterium*-mediated transformation of somatic embryos as a method for the production of transgenic plants. J Tissue Cult Meth 12:145–150
- Dandekar AM, McGranahan GH, Uratsu SL, Leslie CA, Vail PV, Tebbets JS, Hoffmann D, Driver J, Viss P, James DJ (1992) Engineering for apple and walnut resistance to codling moth. In: Proc Brighton Crop Protection Conf on Pests and Diseases, vol 2. British Crop Protection Council, Cambridge, pp 741–747
- Dandekar AM, McGranahan GH, Vail PV, Uratsu SL, Leslie C, Tebbets JS (1994) Low levels of expression of wild type *Bacillus thuringiensis* var *kurstaki* cryIA(c) sequences in transgenic walnut somatic embryos. Plant Sci 96:151–162

- Dandekar AM, McGranahan GH, Vail PV, Uratsu SL, Leslie CA, Tebbets JS (1998) High levels of expression of full-length *cryIA(c)* gene from *Bacillus thuringiensis* in transgenic somatic walnut embryos. *Plant Sci* 131:181–193
- Dandekar AM, Leslie CA, McGranahan GH (2005) *Juglans regia* walnut. In: Litz RE (ed) *Biotechnology of fruit and nut crops*. CABI Publishing, Cambridge, Massachusetts, pp 307–324
- Dangl GS, Woeste K, Aradhya MK, Hoehmstedt A, Simon C, Potter D, Leslie CA, McGranahan GH (2005) Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut. *J Am Soc Hortic Sci* 130:348–354
- Dolcet-Sanjuan R, Claveria E, Gruselle R, Meier-Dinkel A, Jay-Allemand C, Gaspar T (2004) Practical factors controlling in vitro adventitious root formation from walnut shoot microcuttings. *J Am Soc Hortic Sci* 129:198–203
- Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. *HortScience* 19: 507–509
- Duroux L, Delmotte FM, Lancelin JM, Keravis G, Jay-Allemand C (1998) Insight into naphthoquinone metabolism: beta-glucosidase-catalysed hydrolysis of hydrojuglone  $\beta$ -D-glucopyranoside. *Biochem J* 333:275–283
- El Euch C, Jay-Allemand C, Pastuglia M, Doumas P, Charpentier JP, Capelli P, Jouanin L (1998) Expression of antisense chalcone synthase RNA in transgenic hybrid walnut microcuttings: effect on flavonoid content and rooting ability. *Plant Mol Biol* 38:467–479
- Ermel FF, Vizoso S, Charpentier JP, Jay-Allemand C, Catesson AM, Couee I (2000) Mechanisms of primordium formation during adventitious root development from walnut cotyledon explants. *Planta* 211:563–574
- Escobar MA, Park JJ, Polito VS, Leslie CA, Uratsu SL, McGranahan GH, Dandekar AM (2000) Using GFP as a scorable marker in walnut somatic embryo transformation. *Ann Bot* 85:831–835
- Escobar MA, Civerolo EL, Summerfelt KR, Dandekar AM (2001) RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proc Natl Acad Sci USA* 98:13437–13442
- Escobar MA, Leslie CA, McGranahan GH, Dandekar AM (2002) Silencing crown gall disease in walnut (*Juglans regia* L.). *Plant Sci* 163:591–597
- Escobar MA, Civerolo EL, Polito VS, Pinney KA, Dandekar AM (2003) Characterization of oncogene-silenced transgenic plants: implications for *Agrobacterium* biology and post-transcriptional gene silencing. *Mol Plant Pathol* 4:57–65
- Feldman EB (2002) The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. *J Nutr* 132:1062S–1101S
- Forde HL, Griggs WH (1972) Pollination and blooming habits of walnuts. Agriculture Experimental Station, University of California, Leaflet 2753 AXT-N24
- Forde HL, McGranahan GH (1996) Walnuts. In: Janick J, Moore J (eds) *Fruit breeding*. John Wiley, New York, pp 241–273
- Faroni I, Rao R, Woeste K, Gallitelli M (2005) Characterisation of *Juglans regia* L. with SSR markers and evaluation of genetic relationships among cultivars and the 'Sorrento' landrace. *J Hortic Sci Biotechnol* 80:49–53
- Fukuda T, Ito H, Yoshida T (2004) Effect of the walnut polyphenol fraction on oxidative stress in type 2 diabetes mice. *BioFactors* 21:251–253
- Gaspar T, Kevers C, Hausman JF, Berthon JY, Ripetti V (1992) Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. *Agronomie* 12:757–765
- Gillen LJ, Tapsell LC, Patch CS, Owen A, Batterham M (2005) Structured dietary advice incorporating walnuts achieves optimal fat and energy balance in patients with type 2 diabetes mellitus. *J Am Diet Assoc* 105:1087–1096
- Greve JC, McGranahan GH, Hasey J, Snyder R, Kelly K, Goldhamer D, Labavitch JM (1992) Variation in polyunsaturated fatty acids composition of Persian walnut. *J Am Soc Hortic Sci* 117:518–522
- Hammons B, Ponder F, Rickman J (2004) Beyond the wild nut: moving toward profitable black walnut nut crops. In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) *Black walnut in a new century*. Proc 6th Walnut Council Res Symp, Lafayette,

- Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 156–160
- Haque R, Bin-Hafeez B, Parvez S, Pandey S, Sayeed I, Raisuddin S (2003) Aqueous extract of walnut (*Juglans regia* L.) protects mice against cyclophosphamide induced biochemical toxicity. *Human Exp Toxicol* 22:473–480
- Howard WL (1945) Luther Burbank's plant contributions. *Calif Agric Exp Stat Bull* 691
- Jagtap AG, Karkera SG (2000) Extract of *Juglandaceae regia* inhibits growth, in-vitro adherence, acid production and aggregation of *Streptococcus mutans*. *J Pharm Pharmacol* 52:235–242
- Jay-Allemand C, Capelli P, Cornu D (1992) Root development of in vitro hybrid walnut micro-cuttings in a vermiculite-containing gelrite medium. *Sci Hort* 15:335–342
- Kevers C, Bisbis B, Crevecoeur M, Gaspar T, Dommès J (2004) Wood formation in in vitro propagated walnut shoots in relation with root formation and development. *Acta Bot Gall* 151:45–53
- Leslie C, McGranahan G (1992) Micropropagation of Persian walnut (*Juglans regia* L.). In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 18. High-tech and micropropagation II. Springer, Berlin Heidelberg New York, pp 136–150
- Leslie CA, McGranahan GH (1998) The origin of the walnut. In: Ramos DE (ed) *Walnut orchard management*. University of California Division of Agriculture and Natural Resources, Publ 3373, pp 3–7
- Leslie CA, McGranahan GH, Dandekar AM, Uratsu SL, Vail PV, Tebbets JS (2001) Development and field-testing of walnuts expressing the *cryIA(c)* gene for Lepidopteran insect resistance. *Acta Hort* 544:195–199
- Leslie CA, Hackett WP, Bujazha D, Hirbod S, McGranahan GH (2006) Adventitious rooting and clonal plant production of hybrid walnut (*Juglans*) rootstock selections. *Acta Hort* 705:325–328
- López JM (2001) Field behavior of self-rooted walnut trees of different cultivars produced by tissue culture and planted in Murcia (Spain). *Acta Hort* 544:543–546
- Luza JG, Polito VS (1988) Cryopreservation of Persian walnut (*Juglans regia* L.) pollen. *Euphytica* 37:141–148
- Mahoney N, Molyneux RJ, McKenna J, Leslie CA, McGranahan G (2003) Resistance of 'Tulare' walnut (*Juglans regia* cv. Tulare) to aflatoxigenesis. *J Food Sci* 68:619–622
- Manning WE (1978) The classification within the *Juglandaceae*. *Ann Missouri Bot Garden* 65:1058–1087
- Marmioli M, Antonioli G, Maestri E, Marmioli N (2005) Evidence of the involvement of plant ligno-cellulosic structure in the sequestration of Pb: an X-ray spectroscopy-based analysis. *Environ Pollution* 134:217–227
- McGranahan GH, Catlin PB (1987) *Juglans* rootstocks. In: Rom RC, Carlson RF (eds) *Rootstocks for fruit crops*. John Wiley, New York, pp 411–450
- McGranahan GH, Leslie CA (1990) Walnut (*Juglans* L.). In: Moore JN, Ballington JR (eds) *Genetic resources of fruit and nut crops*, vol 2. International Society for Horticultural Science, Wageningen, pp 907–951
- McGranahan GH, Tulecke W, Arulsekhar S, Hansen JJ (1986) Intergeneric hybridization in the *Juglandaceae*: *Pterocarya* sp. × *Juglans regia*. *J Am Soc Hortic Sci* 111:627–630
- McGranahan GH, Driver JA, Tulecke W (1987) Tissue culture of *Juglans*. In: Bonga JM, Durzan DJ (eds) *Cell and tissue culture in forestry*, vol 3. Martinus Nijhoff, Leiden, pp 261–271
- McGranahan GH, Leslie CA, Uratsu SL, Martin LA, Dandekar AM (1988) *Agrobacterium* mediated transformation of walnut somatic embryos and regeneration of transgenic plants. *Bio/Technology* 6:800–804
- McGranahan GH, Leslie CA, Uratsu SL, Dandekar AM (1990) Improved efficiency of the walnut somatic embryo gene transfer system. *Plant Cell Rep* 8:512–516
- Morgan JM, Horton K, Reese D, Carey C, Walker K, Capuzzi DM (2002) Effects of walnut consumption as part of a low-fat, low-cholesterol diet on serum cardiovascular risk factors. *Int J Vit Nutr Res* 72:341–347



- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tissue cultures. *Physiol Plant* 15:473–497
- Navatel JC, Bourrain L (2001) Plant production of walnut *Juglans regia* L. by in vitro multiplication. *Acta Hort* 544:465–471
- Neuman MC, Preece JE, Sambeek JW, Gaffney G (1993) Somatic embryogenesis and callus production from cotyledon explants of Eastern black walnut. *Plant Cell Tissue Organ Cult* 32:9–18
- Ninot A, Aleta N (2003) Identification and genetic relationship of Persian walnut genotypes using isozyme markers. *J Am Pomolog Soc* 57:106–114
- Onay O, Beis S, Kockar OM (2004) Pyrolysis of walnut shell in a well-swept fixed-bed reactor. *Energy Sources* 26:771–782
- Orel G, Marchant AD, McLeod JA, Richards GD (2003) Characterization of 11 *Juglandaceae* genotypes based on morphology, cpDNA, and RAPD. *Hortscience* 38:1178–1183
- Pearson S, Preece JE, Van Sambeek JW (2000) In vitro shoot multiplication of adult black walnut (*Juglans nigra* L.). *HortScience* 35:446
- Pearson S, Preece JE, Van Sambeek JW (2001) Micropropagation of recultured shoots of adult eastern black walnut (*Juglans nigra* L.). *HortScience* 36:584
- Pence VC (1990) Cryostorage of embryo axes of several large-seeded temperate tree species. *Cryobiology* 27:212–218
- Polito VS, McGranahan GH, Pinney K, Leslie CA (1989) Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans regia* L.): implications for *Agrobacterium*-mediated transformation. *Plant Cell Rep* 8:219–221
- Pollegioni P, Bartoli S, Cannata F, Malvolti ME (2003) Genetic differentiation of four Italian walnut (*Juglans regia* L.) varieties by inter simple sequence repeat (ISSR). *J Genet Breed* 57:231–240
- Pollegioni P, Major A, Bartoli S, Ducci F, Proietti R, Malvolti ME, Avanzato D (2006) Application of microsatellite and dominant molecular markers for the discrimination of species and interspecific hybrids in *Juglans* genus. *Acta Hort* 705:191–197
- Potter D, Gao F, Aiello G, Leslie CA, McGranahan GH (2002a) Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars. *J Am Soc Hortic Sci* 127:75–81
- Potter D, Gao F, Baggett S, McKenna JR, McGranahan GH (2002b) Defining the sources of Paradox: DNA sequence markers for North American walnut (*Juglans* L.) species and hybrids. *Sci Hort* 94:157–170
- Preece JE, Van Sambeek JW, Huetteman CA, Gaffney GR (1989) Biotechnology: in vitro studies with walnut (*Juglans*) species: the continuing quest for quality. *Proc 4th Black Walnut Symp*, Carbondale, Illinois
- Preece JE, McGranahan GH, Long LM, Leslie CA (1995) Somatic embryogenesis in walnut (*Juglans regia*). In: Jain S, Gupta P, Newton R (eds) *Somatic embryogenesis in woody plants*, vol 2. Kluwer, Dordrecht, pp 99–116
- Qadan F, Thewaini AJ, Ali DA, Afifi R, Elkhawad A, Matalka KZ (2005) The antimicrobial activities of *Psidium guajava* and *Juglans regia* leaf extracts to acne-developing organisms. *Am J Chin Med* 33:197–204
- Ramos DE (1998) Walnut production manual. University of California Division of Agriculture and Natural Resources, Publ 3373
- Reid W, Coggeshall MV, Hunt KL (2004) Cultivar evaluation and development for black walnut orchards. In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) *Black walnut in a new century*. Proc 6th Walnut Council Res Symp, Lafayette, Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 18–24
- Reiter RJ, Manchester LC, Tan DX (2005) Melatonin in walnuts: influence on levels of melatonin and total antioxidant capacity of blood. *Nutrition* 9:920–924
- Ripetti V, Kevers C, Gaspar T (1994) Two successive media for the rooting of walnut shoots in vitro. Changes in peroxidase activity and in ethylene production. *Adv Hortic Sci* 8:29–32



- Robichaud RL, Glaubitz JC, Rhodes OE, Woeste KE (2006) A robust and powerful set of black walnut microsatellites for parentage and clonal identification. *New Forests* 32:179–196
- Rodriguez R (1982a) Callus initiation and root formation from in vitro culture of walnut cotyledons. *HortScience* 17:195–196
- Rodriguez R (1982b) Stimulation of multiple shoot-bud formation in walnut seeds. *HortScience* 17:592
- Ros E, Nunez I, Perez-Heras R, Serra M, Gilabert R, Casals E, Deulofeu M (2004) A walnut diet improves endothelial function in hypercholesterolemic subjects: a randomized crossover trial. *Circulation* 109:1609–1614
- Saadat YA, Hennerty MJ (2002) Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.). *Sci Hort* 95:251–260
- Setka G (1994) Cryopreservation of walnut (*Juglans regia*) somatic embryos. MSc Thesis, University of California Davis, California
- Shifley SR (2004) The black walnut resource in the United States. In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) *Black walnut in a new century*. Proc 6th Walnut Council Res Symp, Lafayette, Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 168–176
- Smith RE, Smith CO, Ramsey HJ (1912) Walnut culture in California. Walnut blight. University of California Public Bull 231, Berkeley
- Somers PW, Sambeck JW, Preece JE, Gaffney G, Myers O (1982) In vitro micropropagation of black walnut (*Juglans nigra* L.). In: Thielges BA (ed) Proc 7th North American Forest Biology Worksh, University of Kentucky, Lexington, pp 224–230
- Tourjee KR, Gradziel TM (2001) Establishing breeding programmes for new crops: lessons from the eastern black walnut programme. *Outlook Agric* 30:195–203
- Tulecke W, McGranahan GH (1985) Somatic embryogenesis and plant regeneration from cotyledons of walnut *Juglans regia* L. *Plant Sci* 40:57–63
- Tulecke W, McGranahan GH (1988) Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut *Juglans regia* L. cv. 'Manregian'. *Plant Cell Rep* 7:301–304
- Tulecke W, McGranahan GH, Ahmadi H (1988) Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut *Juglans-regia* L. cultivar Manregian. *Plant Cell Rep* 7:301–304
- Tulecke W, McGranahan GH, Leslie CA (1995) Somatic embryogenesis in walnut (*Juglans* species). In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 30. Somatic embryogenesis and synthetic seed, I. Springer, Berlin Heidelberg New York, pp 370–377
- Vahdati K, McKenna JR, Dandekar AM, Leslie CA, Uratsu SL, Hackett WB, Negri P, McGranahan GH (2002) Rooting and other characteristics of a transgenic walnut hybrid (*Juglans hindsii* × *J. regia*) rootstock expressing *rolABC*. *J Am Soc Hortic Sci* 127:724–728
- Vahdati K, Leslie C, Zamani Z, McGranahan G (2004) Rooting and acclimatization of in vitro-grown shoots from mature trees of three Persian walnut cultivars. *HortScience* 39:324–327
- Vail PV, Tebbets JS, Hoffmann DF, Dandekar AM (1991) Response of production and postharvest walnut pests to *Bacillus thuringiensis* insecticidal crystal protein fragments. *Biol Control* 1:329–333
- Victory E, Woeste K, Rhodes OE (2004) History of black walnut genetics research in North America. In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) *Black walnut in a new century*. Proc 6th Walnut Council Res Symp, Lafayette, Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 1–8
- Vyas D, Sharma SK, Sharma DR (2003) Genetic structure of walnut genotype using leaf isozymes as variability measure. *Sci Hort* 97:141–152
- Weinstein JN (2002) 'Omic' and hypothesis-driven research in the molecular pharmacology of cancer. *Curr Opin Pharmacol* 2:361–365
- Whitson J, John R, Williams HS (eds) (1914) *Luther Burbank: his methods and discoveries and their practical application*. Luther Burbank Press, New York

- Woeste KE, McKenna JR (2004) Walnut genetic improvement at the start of a new century. In: Michler CH, Pijut PM, Van Sambeek J, Coggeshall M, Seifert J, Woeste K, Overton R (eds) Black walnut in a new century. Proc 6th Walnut Council Res Symp, Lafayette, Indiana. Gen Tech Rep NC-243. USDA Forest Service, North Central Research Station, St. Paul, Minnesota, pp 9–17
- Woeste K, McGranahan GH, Bernatzky R (1996a) Randomly amplified polymorphic DNA loci from a walnut backcross [*(Juglans hindsii*  $\times$  *J regia*)  $\times$  *J regia*]. J Am Soc Hortic Sci 121:358–361
- Woeste K, McGranahan G, Bernatzky R (1996b) The identification and characterization of a genetic marker linked to hypersensitivity to the cherry leafroll virus in walnut. Mol Breed 2:261–266
- Woeste K, Burns R, Rhodes OE, Michler C (2002) Thirty polymorphic nuclear microsatellite loci from black walnut. J Hered 93:58–60
- Yajima M, Nakamura H, Takahashi K, Watanabe Y, Saito S, Yokozawa Y (1997) Somatic chromosome numbers of colchicines-treated Shinano walnut (*Juglans regia* L.) and its F1 seedlings. J Jpn Soc Hortic Sci 65:677–683
- Yajima M, Watanabe Y, Yanagisawa K, Shomura S, Chino S, Oyamada S, Sato S, Torra-Reventos M, Yamaura I, Yamanaka S (2003) Comparison of pollen characteristics, meiotic division and chromosome pairing between diploid and tetraploid Shinano walnut (*Juglans regia* L. cv. Mitsuru). J Jpn Soc Hortic Sci 72:134–140
- Zambon D, Sabate J, Munoz S, Campero B, Casals E, Merlos M, Laguna JC, Ros E (2000) Substituting walnuts for monounsaturated fat improves the serum lipid profile of hypercholesterolemic men and women: a randomized crossover trial. Ann Internal Med 132:538–546
- Zibaenezhad MJ, Rezaiezadeh M, Mowla A, Ayatollahi SMT, Panjehshahin MR (2003) Antihypertriglyceridemic effect of walnut oil. Angiology 54:411–414

## II.2 Rubber

P. AROKIARAJ<sup>1</sup>

### 1 Introduction

The growth of rubber plantations in Malaya in 1896 was initiated by the progeny of the early collection of *Hevea brasiliensis* seeds, by Henry Wickham from the Amazon jungle (Brazil) in 1876. *Hevea brasiliensis* is a member of the family Euphorbiaceae. Natural rubber comes from the latex, synthesized in specialized cells called laticifers or 'latex vessels', which are formed mainly in the bark, in rings interspersed with the sieve tubes of the secondary phloem of the trunk. Anastomoses between adjacent vessels in each ring allow the latex to drain from a large area of the cortex on tapping the rubber tree (Gomez and Moir 1979).

Latex is a highly specialized cytoplasm containing typical structures, such as nuclei and mitochondria, endoplasmic reticulum and ribosomes, as well as polyisoprene (Gomez 1976). In addition, *Hevea* latex contains two other types of particle, namely lutoid and Frey-Wyssling complex. Lutoid is a major component of latex (Homans and Van Gils 1948) and forms the bulk of the 'bottom fraction' which is sedimented from fresh latex by centrifugation (Moir 1959). It plays an important role in the cessation of latex flow after tapping and, hence, in the determination of latex yield (Boatman 1966). The Frey-Wyssling complex is a minor component of *Hevea* latex, which is a double-membrane organelle containing carotenoid pigments, and is thought to be a highly modified plastid (Dickenson 1965).

### 2 The Malaysian Rubber Industry

The Malaysian rubber industry records a shift of emphasis from the upstream rubber production sector to that of the value-added downstream sectors related to rubber product manufacturing and the timber industry. Nevertheless, the Malaysian government's commitment to increasing yield and the upward trend in rubber prices in 2004 have made possible a 19% increase (compared to 2003) in natural rubber (NR) production to 1.17 million tonnes. This increase

<sup>1</sup>International Islamic University Malaysia, Kulliyah of Science, Department of Biotechnology, Jalan Istana Bandar, Indra Mahkota, 25200 Kuantan, Pahang, Malaysia, e-mail: arokiaraj@iiu.edu.my

was due largely to the smallholding sector, which contributed most of the total production. In 2004, NR exports increased by 16.7% to 1,106,086 tonnes compared with 2003, and trade in rubber products also showed favourable growth. The increased earnings recorded from the export of raw rubber were RM5.20 billion, rubber products RM7.88 billion and rubber wood products RM6.47 billion. Malaysia's trade in the NR sector in 2004 thus continued to be favourable, and the NR industry amounted to RM19.56 billion in export earnings, compared to RM15.02 billion in 2003 (Abdul Hamid 2004). According to the Department of Statistics, Malaysia, and the Malaysian Timber Board (MTIB), the export earnings for the NR industry amounted to RM21.07 billion in 2005.

The natural rubber industry will continue to contribute to Malaysia's export earnings, and the industry should now be viewed as an integral economic entity, covering not only rubber production and the utilization of rubber wood for downstream product manufacturing, but also value-added product derivatives from the rubber tree. In this respect, the Rubber Research Institute of Malaysia has pioneered tissue culture research in *Hevea*, with the primary objective of improving, via genetic transformation, the tree's various agronomic traits and, secondly, adding value to the tree by producing proteins of economic value.

### 3 *Hevea* Tissue Culture and Genetic Transformation

The main challenge for genetic transformation of trees such as *Hevea* is the development of an efficient transformation system for the most desirable cultivars. In this respect, it is not Biolistic transformation per se that is limiting, but the in vitro technology to regenerate complete trees from single cells containing the transgenes. In connection with this, differentially expressed cDNA sequences have been identified in friable *Hevea* callus lines, which relates to its Embryogenesis and regenerative potentials (Charbit et al. 2004). Tissue culture has been employed for the large-scale propagation of clonally identical plants. In the case of *Hevea*, since the traditional method of vegetative propagation is successful for the large-scale generation of clonal plants, tissue culture at the Malaysian Rubber Board (MRB) is directed towards genetic transformation.

Transgenic rubber plants are generated by inserting genes into *Hevea* anther cells and regenerating plants from the transformed cells. Transformation of *Hevea* can be achieved by either direct gene transfer (particle bombardment) or via *Agrobacterium*-mediated delivery. The latter method is preferred for many plants including *Hevea* because of its advantage in producing large numbers of stable transformants. *Agrobacterium*-mediated transformation is the preferred system for the production of transgenic plants, including rubber, as it facilitates whole gene integration with single copy inserts (Arokiaaraj et al. 1998, 2003).

At the MRB, the methodology of culture for genetic transformation is based largely on that adopted by Chen (1984), whereby transformed anther calli are

regenerated into plants via embryogenesis (Wan Abdul Rahaman et al. 1982). The age of the callus employed is critical for *Hevea* genetic transformation. In a study on the effect of age of *Hevea* anther callus (Gl 1 cultivar) on transformation efficiency, 30- to 40-day-old anther callus was shown to be most responsive to *Agrobacterium* infection (Arokiaraj, unpublished data). The selection agent normally used for *Hevea* genetic transformation is kanamycin.

### 3.1 *Agrobacterium*-Mediated Transformation

In many dicotyledonous plants, *Agrobacterium tumefaciens*-mediated transformation has been well established (reviewed in Hooykaas and Schilperroot 1992). The soil bacterium, *A. tumefaciens*, is capable of transferring a defined piece of DNA (T-DNA) from its tumour-inducing (Ti) plasmid into the genome of a large number of gymnosperms and angiosperms. The transfer of T-DNA from *Agrobacterium* to *Hevea* cells takes place when the bacteria are allowed to infect seedlings grown in vitro and in vivo (Arokiaraj and Wan Abdul Rahaman 1991). Various factors affect the success of transformation in the *Agrobacterium*-mediated system, e.g. (1) the interaction between *Agrobacterium* strain and plant genotype, (2) efficient induction of *vir* genes, (3) the type and age of explant and (4) the selection system. In the case of *H. brasiliensis*, several wild-type strains of *A. tumefaciens* (C58 and T37-nopaline type; A348-octopine type and A281-agropine type) are known to induce crown galls, and the tumour growth was most vigorous with C58, followed by T37 and A348 (Arokiaraj 2000). The *Agrobacterium* strain RRIM 541/71, which was isolated by the Rubber Research Institute of Malaysia in 1971 and classified as octopine type, produced a similar response following infection of *Hevea* (Arokiaraj and Wan Abdul Rahaman 1991). *Hevea* also responded well to transformation by derivatives of *Agrobacterium* strains C58 (GV2260 and GV3850) (Arokiaraj et al. 1998, 2002a; Yeang et al. 2002).

An *A. tumefaciens*-mediated transformation procedure for *H. brasiliensis* cultivar Gl 1 was established using anther callus (Arokiaraj et al. 1997a) (depicted in Fig. 2 of Arokiaraj et al. 2004a). Prolonged exposure of the callus to the bacteria should be avoided, because it can cause an overgrowth of bacteria, which is difficult to eradicate. After co-culture, the activity of the reporter gene *uidA* encoding  $\beta$ -glucuronidase (GUS) was detected in transformed Tissue culture (Arokiaraj et al. 1997a) and the latex of transgenic plants (Arokiaraj et al. 1998). The resulting transgenic plants were multiplied by vegetative propagation and the stability of transgenes (*uidA* and *nptII*) was confirmed in the propagated plants (Arokiaraj et al. 1997b).

Attempts have also been made to improve the transformation frequency for *Hevea*. It was reported that gene transfer efficiency to plant cells is improved significantly by the use of the supervirulent plasmid pToK47 (Jin et al. 1987). The supervirulence characteristic may be attributed to enhanced transcription of the *vir* genes, thereby facilitating more efficient transport

of the T-strand through the bacterial cell wall. The *Agrobacterium* strains GV2260(pLGMR.HSA) and GV3850(pGPTV-ScFv4715) containing pToK47 appear to be more efficient for *Hevea* transformation (Arokiaraj, unpublished data). Recently, Blanc and co-authors (2006) reported an efficient procedure for generating transgenic callus and plants from *Hevea* clone PB260 using cryopreserved callus in combination with reduced cocultivation temperature from 27 to 20 °C.

Whilst the *Hevea* clone Gl 1 has been commonly used in genetic transformation in Malaysia, recent research has utilized other high-yielding *Hevea* cultivars (Thulaseedharan 2002; Montoro et al. 2003). For example, in India, transgenic rubber clone RRII 105 has been reported expressing the superoxide dismutase (SOD) gene (Sobha et al. 2003; Jayashree et al. 2004).

### 3.2 Biolistic Transformation

Biolistic transformation, also known as particle bombardment, was developed by Sanford et al. (1987), and is a physical gene transfer method of introducing genes into target plant materials. Using this procedure, numerous plant species have been transformed (Klein et al. 1992; Sanford et al. 1993). Particle bombardment is also used as a means to obtain stable transgenic woody perennials, including cotton (McCabe and Martinell 1993) and yellow poplar (Wilde et al. 1992). In fact, the first genetic transformation of *H. brasiliensis* callus was carried out by particle bombardment (Kitayama et al. 1990), but transgenic plants were not reported. Arokiaraj et al. (1994) reported the first transgenic rubber plant expressing *gus* following particle bombardment.

## 4 Rubber Trees as Recombinant Protein Factories

The adoption of plants as biofactories for the production of recombinant proteins is based on their cost-effectiveness. In this regard, a major determinant lies in efficient recovery of the synthesized protein. In general, the recombinant protein harvested from a transgenic plant involves destruction of the plant or a portion of it, regardless of whether the target protein is present in seeds, leaves or shoots. In this instance, the harvested protein follows a batch-wise production, rather than an economical continual process.

Rubber has a distinct advantage over many other plant species, as it produces a large amount of latex. The latex that is synthesized is stored in vessels termed 'latex vessels', which are localized in the outer bark, overlaying the cambium (Dickenson 1969). When the rubber tree is tapped, the latex exudes from the Laticifer, latex vessels and, therefore, transgenic *Hevea* plants can provide a non-destructive system for harvesting recombinant proteins that are synthesized in the latex. Transgenic *Hevea* thus serves as a mini plant factory or bioreactor for recombinant protein production. Continual production of

the recombinant proteins is possible, as the tree can be tapped every other day for its latex throughout its life-span of 25 years. Furthermore, since *Hevea* latex is free of animal and human viruses, it can be an important resource for the production of therapeutic and healthcare proteins (Yeang et al. 1998; Arokiaraj et al. 2002b). In addition, the ease of extraction of the target protein and the large numbers of high expressing rubber plants that can be easily generated without chimaerism through the routine horticultural practice of bud-grafting make this an attractive system.

Although transgenes can be inserted into the rubber genome, this does not mean that their translation products can be synthesized. Furthermore, these proteins usually undergo post-translational modification, such as glycosylation, which involves the addition of sugar molecules to the protein backbone. The *Hevea* laticiferous system provides the glycosylation machinery, but it is also important to determine whether the glycosylated recombinant proteins follow the same pattern as the native proteins. At MRB, transgenic *Hevea* plants have been expressing the GUS protein (Arokiaraj et al. 1998) and a mouse antibody fragment against the coat protein of an oral dental bacterium, *Streptococcus sanguis*, in their latex (Yeang et al. 2002). The recombinant GUS protein synthesized in the latex exhibited its functionality when supplied with X-Gluc, while the antibody fragment was shown to be immunoreactive to the coat protein of *S. sanguis*.

#### 4.1 Expression Levels of Recombinant Proteins in *Hevea* Latex

For commercial production of recombinant proteins, the rate of production should be high and it should be cost-effective. However, there is considerable variation in the expression levels of recombinant proteins derived from transgenic plants and often this is a problem perceived by plant molecular biologists. Generally, recombinant proteins expressed in transgenic plants have made up less than 1% of total soluble proteins, a level thought to be required for purification with commercial feasibility (Kusnadi et al. 1997). In the case of antibodies, however, it is profitable to produce them at 0.1% of total soluble proteins (Hood et al. 2002). In *Hevea*, expression of the single chain variable fragment antibody (ScFv) in latex serum was 0.02% of the total protein (Yeang et al. 2002) compared to 0.01–1.0% reported in other plant tissues (Table 1). The levels of several recombinant human proteins produced in plants are shown in Table 2. Although chloroplast transformation appears to be the most efficient system for the synthesis of recombinant proteins, the capacity of the chloroplast genome to process many post-translation modifications required for the recombinant proteins is questionable (Twyman et al. 2003). The most common approach is to target the expression of transgenes in the nuclear genome, which has been used to produce several recombinant human proteins in plants ranging from 0.01 to 0.3% of the total proteins. The results of our study showed that transgenic *Hevea* expressed recombinant human serum albumin in the



**Table 1.** Expression levels of single chain variable fragment antibody (scFv) in various plant tissues (Arokiaraj et al. 2004a)

Host plant (organ or tissue)	scFv protein concentration (% total soluble proteins)	Reference
Tobacco (leaf)	0.01–1	Schouten et al. (1996)
Tobacco (seed)	0.67	Fiedler and Conrad (1995)
Tobacco (leaf)	0.5	Firek et al. (1993)
Potato (roots)	0.03–0.3	Schouten et al. (1997)
Tobacco (protoplast)	0.02–0.1	Schouten et al. (1997)
<i>Hevea</i> (latex serum)	0.02	Yeang et al. (2002)
Tobacco (leaf)	0.014	Longstaff et al. (1998)
Tobacco (leaf)	0.01	Bruyns et al. (1996)

latex in concentrations of up to 0.3% of total soluble proteins (Arokiaraj et al. 2002a). Recombinant human serum albumin produced in *Hevea* is greater than in potato, but lower than in the chloroplast transformation system (Table 2).

It has been reported that the production rate of  $150 \text{ mg l}^{-1}$  of recombinant human serum albumin can be achieved using *Pichia pastoris* in a bioreactor (Qiu et al. 2000), which is 4.5-fold higher than that in *Hevea* (Arokiaraj et al. 2002a). Furthermore, a production rate exceeding 10 g of human serum albumin has been recorded (Kobayashi 2000). From a commercial viewpoint, achieving a high production rate of recombinant protein is an important factor for the success of using transgenic plants as bioreactors. This may be accomplished by the use of tissue-specific promoters and related sequences to target gene expression in compartmentalized areas in plants. In *Hevea*, hevein is a choice promoter for over-expression of recombinant proteins in latex, as hevein is the most abundant soluble protein. The signal sequences that are responsible for improved hevein expression in response to wounding, ethylene or abscisic acid are located in the 5' upstream regulatory region (Broekaert et al. 1990). The hevein gene and its promoter sequences have been isolated (GenBank Accession no. AF287016, Arokiaraj and Jones 2001; AF327518) and characterized (Deng et al. 2002). In addition to hevein, the *hevea* glutamine synthase (GS) promoter has been shown to confer *uidA* expression in *Hevea* callus (Pujade-Renaud et al. 2000). Similar transgene expression conferred by hevein and GS promoters has also been reported in *Hevea* callus and somatic embryos of tobacco (Pujade-Renaud et al. 2003, 2005; Arokiaraj et al. 2002c, 2004b).

## 4.2 Stability of Transgene Expression

As *Hevea* is a perennial, it is important to ensure stable expression of transgenes during the life-span of the tree. Stable transgene expression is also essential during vegetative propagation of transgenic plants by budding. However, in-

**Table 2.** Expression levels of recombinant human proteins in plant tissues (Arokiaraj et al. 2004a). Data for recombinant human serum albumin expression are in bold for comparison

Recombinant protein	Gene source	Host plant (organ or tissue)	Recombinant protein concentration (% of total soluble proteins)	Reference
Serum albumin	Human	Tobacco (chloroplast)	11.1	Fernández-San Millan et al. (2003)
Somatotropin (growth hormone)	Human	Tobacco (chloroplast)	7.0	Staub et al. (2000)
Serum albumin	Human	<i>Hevea</i> (latex serum)	0.3	Arokiaraj et al. (2002b)
Hirudin	Human	Canola (seed)	0.3	Cramer et al. (1999)
Enkephalins	Human	<i>Arabidopsis</i> (seed)	0.1	Vandekerckhov et al. (1989)
Lactoferrin	Human	Potato (tuber)	0.1	Chong and Langridge (2000)
Haemoglobin $\alpha, \beta$	Human	Tobacco	0.05	Cramer et al. (1999)
Serum albumin	Human	Tobacco (cell suspension culture medium)	0.025	Sijmons et al. (1990)
		Potato (leaves)	0.02	
Epidermal growth factor	Human	Tobacco	< 0.01	Higo et al. (1993)
Erythropoietin	Human	Tobacco	< 0.01	Kusnadi et al. (1997)
Protein C	Human	Tobacco	< 0.01	Cramer et al. (1999)

formation regarding the stability of transgene expression in *Hevea* during vegetative propagation has been limited. We previously showed that the two transgenes, *nptII* and *gus*, continued to be expressed in leaves of vegetatively propagated plants (Arokiaraj et al. 1997b). GUS expression was also detected in latex of 200 bud-derived plants from the original transformants (Arokiaraj 2000). The GUS protein was detected in latex of three vegetative generations of rubber trees and also in embryo-rescued seedlings (Arokiaraj et al. 2003, 2004b). These results indicate that transgene expression in *Hevea* is relatively stable. This is in line with the ELISA assays, where transgene expression in latex of the mouse antibody fragment (ScFv4715) against the coat protein of the oral dental bacterium *S. sanguis* was stable in transgenic *Hevea* after vegetative propagation (Arokiaraj et al. 2003). From a number of transgenic plants that have been produced, those showing the strongest functional protein can be exploited in order to generate vegetatively a large number of transformants showing transgene stability and expression.

## 5 Molecular Genetics for *Hevea* Improvement

Over the last 70 years, significant effort has been directed at rubber tree improvement through conventional breeding. This has resulted in trees yielding latex and timber and classified as latex timber clones. However, further improvement may be introduced using endogenous or heterologous genes to improve the agronomic traits of the rubber tree by genetic engineering. This would help to reduce the time required for *Hevea* Genetic improvement and become an important part of *Hevea* breeding programmes. As genetic transformation procedures are developed for *Hevea*, they can be used to generate novel *Hevea* trees with high rubber productivity and timber, the development of high-yielding dwarf clones to negate the consequences of wind damage and contribute to high-density planting (Venkatachalam et al. 2004), or resistance to pathogens by metabolic engineering.

### 5.1 Latex Yield

In *Hevea*, rubber formation emerges from a main pathway where acetyl CoA is converted to mevalonic acid (MVA) and isopentenyl pyrophosphate, and to long chain prenyl diphosphates. The reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG) to mevalonic acid is catalyzed by the enzyme HMG-CoA reductase (HMGR; EC 1.1.1.34), which is an irreversible reaction. HMGR in *Hevea* latex has been characterized and the activity appears to be exceptionally low (0.078 nmol MVA/ml of latex), compared to that of other enzymes (Lynen 1969). Thus, the level of this enzyme may be a limiting factor in rubber biosynthesis. Similar results were also observed when mevalonic acid was utilized by latex at a much faster rate than HMG-CoA (Hepper and Audley 1969). Wititsuwannakul (1986) reported that the high specific activity of HMGR was obtained from high-yielding *Hevea* clones, and that the rubber content in the latex varied diurnally in a pattern similar to the variation in HMGR-CoA reductase activity. It is therefore possible to increase rubber yield by promoting transcription and translation of latex-specific HMGR.

The HMGR genes isolated from *Hevea* were encoded by a small gene family consisting of three members, *hmgr1*, *hmgr2* and *hmgr3* (Chye et al. 1991, 1992). *hmgr1* was predominantly expressed in laticifer tissue and was inducible by ethylene, whereas *hmgr3* was constitutively expressed. In a study where *hmgr1* was transferred to *Hevea* anther callus by particle bombardment, HMGR activity in transformed calli and somatic embryos was shown to increase by up to 580% compared with control cultures (Arokiaraj 1995). This observation showed that the activity of a key enzyme in rubber biosynthesis could be elevated through genetic transformation.

It has long been established that the number of laticifers is one of the most important factors influencing latex yield in *Hevea* (Gomez 1982). Laticifer ring differentiation from vascular cambium in *Hevea* clones is genetically controlled

by environmental conditions. Furthermore, latex exploitation also influences the formation of laticifer rings (Hao and Wu 2000), as exploited trees produced two to three times the number of laticifer rings in the same period in the area of bark from which the latex flowed compared to untapped trees. According to Hao and Wu (2000), jasmonic acid is the signaling molecule involved in laticifer differentiation. This signaling molecule is synthesized in plants from linolenic acid via the octadecanoid pathway, similar to the pathway in the synthesis of eicosanoids in animals (Creelman and Mullet 1997). It is now clear that jasmonic acid and 12-oxophytodienoic acid (OPDA), the first cyclic metabolite in the pathway leading to jasmonic acid synthesis, are important in laticifer differentiation. The first step in jasmonic acid biosynthesis is the conversion of 13(S)-hydroxylinolenic acid to allene oxide, 12,13-epoxytrienoic acid, a reaction catalyzed by the soluble, plastidic cytochrome P450 enzyme allene oxide synthase (AOS). In view of this, the cloning and characterization of *Hevea aos* and *opr* genes (Gareth et al. 2003) will be an important step towards the production of novel transgenic *Hevea* with increased latex yield by modulation of expression of these genes.

## 5.2 Towards Tolerance to Tapping Panel Dryness

A phenomenon most commonly observed in rubber trees due to frequent exploitation and stimulation is tapping panel dryness (TPD). In this case, the luteoids in the latex of exploited trees are affected by interactive disfunctioning of superoxide dismutase (SOD), leading to destabilization of latex. SOD has been considered to assist in the maintenance of luteoid membrane integrity (Chrestin 1989). The high level of free radicals (superoxides) in dry trees, due to abnormally high NAD(P)H oxidase activity, damages the luteoid membrane; in normal trees, these free radicals are mopped up by SOD to provide protection against damage to luteoid membranes (Chrestin 1989). In an attempt to reduce free radicals in the bark of rubber trees that are prevalent to dryness, the Rubber Research Institute of India recently cloned the *sod* gene from *Hevea*, and the homologous version of the gene was introduced into *Hevea* via *Agrobacterium* (Sobha et al. 2003). Transgenic rubber plants were generated via somatic embryogenesis, but the role of SOD in transgenic *Hevea* has yet to be elucidated. Preliminary results of another study demonstrated that transgenic plants expressing the *sod* gene showed increased SOD activity in response to stress (Thulaseedharan 2002).

## 5.3 Increasing the Biomass of Rubber Trees for Early Maturity and Timber Productivity

In general, rubber trees are usually tapped when their girth size reaches about 50 cm and this takes about 5–6 years. The immaturity period can be further reduced to 4 years by using advanced planting materials and good agro-

management practices. In order to apply biotechnological methods to *Hevea* breeding for wood enhancement, genes to be identified include those expressed in wood-forming tissues and those involved in regulating various aspects of wood formation.

To date, the biochemical and physiological processes underlying wood formation and the genes involved are mainly unknown. Two genes that act as developmental switches in xylogenesis of hybrid aspen were isolated by Hertzberg and Olsson (1998). This class of genes, homeobox genes (*PttHB*), encode transcription factors that are expressed specifically in the maturing xylem zone of hybrid aspen. Amongst the two homeobox genes, *PttHB1* was expressed specifically in the xylem maturation zone, indicating a possible role in the regulation of secondary wall formation. This led to the speculation that the *PttHB1* protein is involved in triggering a developmental switch, initiating secondary wall formation in the xylem maturation zone, and leading to stem girth increase in plants. Thus, a similar homologous homeobox gene from *Hevea* could serve as a candidate for increasing the rate of girdling in transgenic *Hevea*.

Gibberellins (GAs) are a class of phytohormones that can influence plant growth and development. It has been demonstrated through hormone application studies that GAs can indeed influence the longitudinal and radial growth of hardwood and conifer species (Hedden and Kamiya 1997). This has been supported by a study in which hybrid aspen trees were transformed with a GA synthesis gene (*ga20 oxidase* cDNA) from *Arabidopsis thaliana* (Eriksson et al. 2000). Transgenic trees were shown to produce high concentrations of GAs and growth rates and increased biomass production, with more numerous and longer stem fibres than unmodified wild-type trees. Furthermore, transgenic trees showed increased shoot elongation and stem diameter. The approach for hybrid aspen can also be employed for *Hevea* improvement to achieve faster growth rates and increased stem girth (Arokiaraj et al. 2002d).

There are numerous enzymes involved in diverting secondary metabolites to the wood-forming process. Research in this area has identified a group of enzymes, the 4-Coumarate – CoA ligases (4-CLs) – as being important in lignin synthesis in the developing xylem tissues of hybrid aspen. For instance, varying concentrations of a specific 4-CL enzyme in transgenic hybrid aspen caused modified phenotypes, including altered stem diameters and xylem cell size (Hu et al. 1998). Thus, by simply altering specific enzymes involved in the wood-forming process, rubber trees could be generated for early maturity with improved timber production.

## 6 Biosafety Aspects of Transgenic *Hevea*

The incidence of transgenes from genetically modified (GM) field trials transferring into natural habitats or non-transgenic crops depends on a number of factors, but of them, the most important is gene flow via pollen transfer.

Therefore, in handling transgenic rubber, the Malaysian Rubber Board takes into account biosafety considerations.

When *Hevea* was introduced into Malaysia from the Amazon, many new rubber plantations were established out of existing jungle and hence located adjacent to jungle areas. Despite this, after more than 100 years of rubber cultivation in Malaysia, feral rubber has not established in Malaysian jungles. This difference is ascribed to differences in the availability of invadable habitats, and the probability of transgenic *Hevea* becoming established will depend largely on the competitiveness of the transgenic trees in their new environment.

Transfer of genes from transgenic *Hevea* via pollen dispersal will depend on the degree of sexual compatibility between the genetically modified species and the selected clones that are vegetatively propagated, and the opportunities for pollination and seed set. In the case of *Hevea*, the frequency of pollination depends on the isolation in space between the genetically modified plants and suitable recipients, depending on insect and wind pollination and flowering season. To clarify this, a study was carried out by Yeang and Chevallier (1999) to determine the rates of cross-pollination by *Hevea* pollen at various distances from the boundary between two adjacent fields that were each planted with a pure stand of a genetic clone. Using esterase isozyme markers to determine if the seeds had been derived from self- or cross-pollination, the authors showed that the movement of genes is negligible beyond 1.1 km, and this is consistent with the low rates of cross-pollination found in *Hevea*.

Pollination of *Hevea* is normally carried out by midges and thrips (Warmke 1951, 1952) and since neither can fly long distances, long-distance pollen dispersal is not expected. However, in cases where pollen escape may occur, such escape is not expected to exert a significant impact on gene flow, because *H. brasiliensis* has a relatively low rate (3%) of fruit-set (Ghandimathi and Yeang 1984).

## 7 Conclusions and Future Challenges

The ability to produce transgenic rubber trees paves the way for the production of a wide range of useful products with considerable potential in the pharmaceutical, cosmetic and medical industries, as reviewed by Yeang et al. (1998) and Arokiaraj et al. (2004a). The utilization of rubber trees as bioreactors for commercial exploitation in the future depends largely on the improvement of transgene expression to a level that makes the system economically viable. To achieve this, it is important to target both gene expression in the specific tissues or organs, by the use of tissue or organ-specific promoters, e.g. latex-specific promoters, and gene expression in special organelles such as luteoids. Optimization of gene expression, coupled with improved genetic transformation, will also be crucial for the improvement of agronomic traits in *Hevea*. These include production of novel plants with improved latex, increased tim-

ber yield, high capacity for carbon sequestration and resistance to disease and environmental stress.

**Acknowledgements.** The author thanks the Director General of the Malaysian Rubber Board and the Dean, Kuliyiyah of Science, International Islamic University Malaysia for granting permission to publish this work.

## References

- Abdul Hamid S (2004) Annual report. Malaysian Rubber Board, Kuala Lumpur
- Arokiaaraj P (1995) Towards molecular genetic improvement of rubber yield in transgenic *Hevea brasiliensis* Muell Arg. PhD Thesis, University of London
- Arokiaaraj P (2000) Genetic transformation of *Hevea brasiliensis* (rubber tree) and its applications towards crop improvement and production of recombinant proteins of commercial value. In: Jain SM, Minocha SC (eds) Molecular biology of woody plants, vol 2. Kluwer, Dordrecht, pp 305–326
- Arokiaaraj P, Jones H (2001) Identification of regulatory sequence in the 5' upstream region of hevein gene from *Hevea brasiliensis*. In: Proc IRRDB Symp on Biotechnology and Rubber Tree, CIRAD, Montpellier, pp 116–118
- Arokiaaraj P, Wan Abdul Rahaman WY (1991) *Agrobacterium* mediated transformation of *Hevea* cells derived from in vitro and in vivo seedling cultures. J Nat Rubb Res 6:55–61
- Arokiaaraj P, Jones H, Cheong KF, Coomber S, Charlwood BC (1994) Gene insertion into *Hevea brasiliensis*. Plant Cell Rep 13:425–431
- Arokiaaraj P, Hafsah J, Cheong KF, Jafri S, Chew NP, Yeang HY (1997a) Sustained activity of inserted GUS gene over four vegetative generations of transgenic *Hevea*. In: Proc 9th National Biotechnology Seminar, Penang, pp 59–63
- Arokiaaraj P, Jones H, Jaafar H, Coomber S, Charlwood BV (1997b) *Agrobacterium*-mediated transformation of *Hevea* anther callus and their regeneration into plantlets. J Nat Rubber Res 11:77–87
- Arokiaaraj P, Yeang HY, Cheong KF, Hamzah S, Jones H, Coomber S, Charlwood BV (1998) CaMV 35S promoter directs  $\beta$ -glucuronidase expression in the laticiferous system of transgenic *Hevea brasiliensis* (rubber tree). Plant Cell Rep 17:621–625
- Arokiaaraj P, Rueker F, Oberyemayr E, Shamsul Bahri AR, Hafsah J, Carter DC, Yeang HY (2002a) Expression of human serum albumin in transgenic *Hevea brasiliensis*. J Rubber Res 5:157–166
- Arokiaaraj P, Rueker F, Oberyemayr E, Shamsul Bahri AR, Hafsah J, Carter DC, Yeang HY (2002b) Towards molecular biopharming using rubber trees. In: Proc BioMalaysia 2002, Int Biotechnol Symp, Exhibition and BioPartnering, Kuala Lumpur
- Arokiaaraj P, Yeang HY, Hafsah J, Arif SAM, Shamsul Bahri AR, Badrul Ezam B (2002c) Prospects and recent developments in *Hevea* genetic transformation at MRB. In: Proc Rubber Planters' Conf, Kottayam, pp 141–145
- Arokiaaraj P, Jones H, Olsson O, Wan Abdul Rahaman WY (2002d) Towards molecular genetic improvement of wood and latex production in *Hevea brasiliensis*: enhancement of the carbon sink capacity. In: Proc 5th Joint Worksh Secretariat of United Nations Conf on Trade and Development and International Rubber Study Group on Rubber and the Environment, Glasgow, pp 1–9
- Arokiaaraj P, Shamsul Bahri AR, Siti Hawa S, Hafsah J (2003) Field evaluation of transgene expression in vegetative generations and embryo-rescued transgenic *Hevea brasiliensis*. In: Proc International Rubber Research Development Board Symp 'Challenges for Natural Rubber in Globalization', Chiang Mai
- Arokiaaraj P, Hafsah J, Yeang HY, (2004a) Genetic transformation of *Hevea brasiliensis*. Malaysian Rubber Board, Kuala Lumpur, Monograph 18, p 5



- Arokiaaraj P, Abdul Razak SB, Jaafar H, Mad Arif SA, Yeang HY (2004b) Transgenic rubber trees: where are we now? Proc International Rubber Research Development Board Worksh, Kuala Lumpur, pp 60–62
- Blanc G, Baptiste C, Oliver G, Martin F, Montoro P (2006) Efficient *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli and regeneration of *Hevea brasiliensis* Müll Arg. plants. Plant Cell Rep 24:724–733
- Boatman SG (1966) Preliminary physiological studies on the promotion of latex flow by plant growth regulators. J Rubber Res Inst Malaya 19:243–258
- Broekaert W, Lee H, Kush A, Chua N-H, Raikhel N (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc Natl Acad Sci USA 87:7633–7637
- Bruyins AM, de Jaeger G, de Neve M, de Wilde C, van Montagu M, Depicker A (1996) Bacterial and plant-produced scFv proteins have similar antigen-binding properties. FEBS Lett 386:5–10
- Charbit E, Legavre T, Lardet L, Bourgeois E, Ferrière N, Carron MP (2004) Identification of differentially expressed cDNA sequences and histological characteristics of *Hevea brasiliensis* calli in relation to their embryogenic and regenerative capacities. Plant Cell Rep 22:539–548
- Chen Z (1984) Rubber (*Hevea*). In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol 2. Crop species. Macmillan, New York, pp 546–571
- Chong DK, Langridge WH (2000) Expression of full-length bioactive antimicrobial human lactoferrin in potato plants. Transgenic Res 9:71–78
- Chrestin H (1989) Biochemical aspects of bark dryness induced by overstimulation of rubber trees with Ethrel. In: Auzac JD, Jacob J-L, Chrestin H (eds) Physiology of rubber tree latex. CRC Press, Boca Raton, pp 431–441
- Chye M-L, Kush A, Tan C-T, Chua N-H (1991) Characterisation of cDNA and genomic clones encoding 3-hydroxy-3-methylglutaryl co enzyme A reductase from *Hevea brasiliensis*. Plant Mol Biol 16:567–577
- Chye M-L, Tan C-T, Chua N-H (1992) Three genes encode 3-hydroxy-3-methylglutaryl co enzyme A reductase in *Hevea brasiliensis*: *hmg1* and *hmg3* are differentially expressed. Plant Mol Biol 19:473–484
- Cramer CL, Boothe J, Oishi KK (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Microbiol Immunol 240:95–117
- Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol 48:355–381
- Deng XD, Fei XW, Huang JS, Zheng XQ (2002) Isolation and analysis of rubber hevein gene and its promoter sequence. Acta Bot Sin 44:936–940
- Dickenson PB (1965) Ultrastructure of latex vessels of *Hevea brasiliensis*. In: Mullins L (ed) Proc Natl Rubber Prod Res Assoc Jubilee Conf, Maclaren, Cambridge, pp 52–66
- Dickenson PB (1969) Electron microscopical studies of the latex vessel system of *Hevea brasiliensis*. J Rubber Res Inst Malaya 21:543–559
- Eriksson ME, Israelsson M, Olsson O, Moritz T (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fibre length. Nat Biotechnol 18:784–788
- Fernández-San Millán A, Mingo-Castel A, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol J 1:71–79
- Fiedler U, Conrad U (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. Bio/Technology 13:1090–1093
- Firek S, Draper J, Owen MR, Gandeche A, Cockburn B, Whitelam GC (1993) Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. Plant Mol Biol 23:861–870
- Gareth N, Jones H, Griffiths D, Stanbury D, Arokiaaraj P, Yusoff F, Yeang HY (2003) Molecular cloning of major enzymes involved in the octadecanoid pathway in *Hevea brasiliensis*. In: Proc 7th Int Congr Plant Molecular Biology, Barcelona, Abstract S26-52

- Ghandimathi H, Yeang HY (1984) The low fruit set that follows hand pollination in *Hevea brasiliensis*: insufficiency of pollen as a cause. *J Rubber Res Inst Malaysia* 32:20–29
- Gomez JB (1976) Comparative ultracytology of young and mature latex vessels in *Hevea brasiliensis*. In: *Proc Int Rubber Conf 1975*, Kuala Lumpur, pp 143–164
- Gomez JB (1982) Anatomy of *Hevea* and its influence on latex production. In: *MRRDB Monograph 7*. Malaysian Rubber Research Development Board, Kuala Lumpur, p 26
- Gomez JB, Moir GFJ (1979) The ultracytology of latex vessels in *Hevea brasiliensis*. In: *MRRDB Monograph 4*. Malaysian Rubber Research and Development Board, Kuala Lumpur, pp 3–4
- Hao B-Z, Wu J-L (2000) Laticifer differentiation in *Hevea brasiliensis*: induction by exogenous jasmonic acid and linolenic acid. *Ann Bot* 85:37–47
- Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. *Annu Rev Plant Physiol Plant Mol Biol* 48:431–460
- Hepper CM, Audley BG (1969) The biosynthesis of rubber from 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Hevea brasiliensis*. *Biochem J* 114:379–386
- Hertzberg M, Olsson O (1998) Molecular characterisation of a novel plant homeobox gene expressed in the maturing xylem zone of *Populus tremula* × *tremuloides*. *Plant J* 16:285–295
- Higo K, Saito Y, Higo H (1993) Expression of a chemically synthesized gene for human epidermal growth factor under the control of cauliflower mosaic virus 35S promoter in transgenic tobacco. *Biosci Biotechnol Biochem* 57:1477–1481
- Homans LNS, Van Gils GE (1948) Fresh *Hevea* latex. A complex colloidal system. In: Dawson TR (ed) *Proc 2nd Rubber Technol Conf*, Heffer, Cambridge, pp 292–302
- Hood EE, Woodward SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants – myths and realities. *Curr Opin Biotechnol* 13:630–635
- Hooykaas PJJ, Schilperoot RA (1992) *Agrobacterium* and plant genetic engineering. In: Schilperoot RA, Dure L (eds) *10 Years molecular biology*. Kluwer, Dordrecht, pp 15–38
- Hu WJ, Kawaoka A, Tsai CJ, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). *Proc Natl Acad Sci USA* 95:4507–5412
- Jayashree R, Rekha K, Venkatachalam P, Uratsu SL, Dandekar AM, Kumari Jayasree P, Kala RG, Priya P, Sushma Kumari S, Sobha S, Ashokan MP, Sethuraj MR, Thulaseedharan A (2004) Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Plant Cell Rep* 22:201–209
- Jin S, Komari T, Gordon MP, Nester EW (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. *J Bacteriol* 169:4417–4425
- Kitayama M, Takahashi M, Surzycki SJ, Togasaki RK (1990) Transformation of callus tissue from *Hevea brasiliensis* and *Jasminium officinale*. *Plant Physiol (Suppl)* 93:46
- Klein TM, Arentzen R, Lewis PA, Fitzpatrick-McElligott S (1992) Transformation of microbes, plants and animals by particle bombardment. *Bio/Technology* 10:286–291
- Kobayashi K (2000) Production of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris*. *Downstream* 31, Amersham Biosciences Customer Magazine 5
- Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. *Biotechnol Bioeng* 56:473–484
- Longstaff M, Newell CA, Boonstra B, Strachan G, Learmonth D, Harris WJ, Porter AJ, Hamilton WD (1998) Expression and characterisation of single-chain antibody fragments produced in transgenic plants against the organic herbicides atrazine and paraquat. *Biochim Biophys Acta* 1381:147–160
- Lynen I (1969) Biochemical problems of rubber synthesis. *J Rubber Res Inst Malaya* 21:389–406
- McCabe DE, Martinell BJ (1993) Transformation of elite cotton cultivars via particle bombardment of meristems. *Bio/Technology* 11:596–598
- Moir GFJ (1959) Ultracentrifugation and staining of *Hevea* latex. *Nature* 184:1626–1628
- Montoro P, Rattana W, Pujade-Renaud V, Michaux-Ferrière N, Monkolsook Y, Kanthapura R, Adunsadthapong S (2003) Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. *Plant Cell Rep* 21:1095–1102

- Pujade-Renaud V, Montoro P, Kongsawadworakul P, Romruensukharom P, Narangajavana J, Chrestin H (2000) Cloning of potentially ethylene-inducible and/or laticifer-specific promoters from *Hevea brasiliensis*. In: Proc 6th Int Congr Plant Molecular Biology, Quebec, Abstract S26-21
- Pujade-Renaud V, Arokiaaraj P, Jones H, Sanier C, Cambillau L, Tharreau D, Narangajavana J, Chrestin H (2003) Functional analysis of promoter sequences from *Hevea brasiliensis* hevein genes in rice and rubber tree. In: Proc 7th Int Congr Plant Molecular Biology, Barcelona, Abstract S05-32
- Pujade-Renaud V, Sanier C, Cambillau L, Arokiaaraj P, Jones H, Ruengsri N, Chrestin H, Tharreau D, Montoro P, Narangajavana J (2005) Molecular characterization of new members of the *Hevea brasiliensis* hevein multigene family and analysis of their promoter region in rice. *Biochim Biophys Acta* 1727:151–161
- Qiu R-D, Li S-Y, Chen J-G, Wu S-F, Yuan Z-Y (2000) High expression and purification of recombinant human serum albumin from *Pichia pastoris*. *Sheng Wu Huo Xue Yu Sheng Wu Wu Xue Bao* 32:59–62
- Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. *J Part Sci Technol* 5:27–37
- Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. *Methods Enzymol* 217:483–509
- Schouten A, Roosien J, van Engelen FA, de Jong GA, Borst-Vrens AW, Zilverentant JF, Bosch D, Stikema WJ, Gommers FJ, Bakker J (1996) The c-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol Biol* 30:781–793
- Schouten A, Roosien J, de Boer JM, Wilmink A, Rosso M-N, Bosch D, Stikema WJ, Gommers FJ, Bakker J, Schots A (1997) Improving scFv antibody levels in the plant cytosol. *FEBS Lett* 415:235–241
- Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. *Biotechnology* 8:217–221
- Sobha S, Sushamakumari S, Thanseem I, Jayasri PK, Rekha K, Jayashree R, Kala RG, Asokan MP, Sethuraj MR, Dandekar AM, Thulaseedharan A (2003) Genetic transformation of *Hevea brasiliensis* with the gene coding for superoxide dismutase with FMV 34S promoter. *Curr Sci* 85:1767–1773
- Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat Biotechnol* 18:333–338
- Thulaseedharan A (2002) Biotechnological approaches for crop improvement in natural rubber at RRII – present status. In: Proc Rubber Planters' Conf, India, pp 135–140
- Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. *Trends Biotechnol* 21:570–578
- Vandekerckhove J, Van Damme J, Van Lijsebettens M, Botterman J, De Block M, Vandewiele M, De Clercq A, Leemans J, Van Montagu M, Krebbers E (1989) Enkephalins produced in transgenic plants using modified 2S seed storage proteins. *Bio/Technology* 7:929–932
- Venkatachalam P, Priya P, Saraswathy Amma CK, Thulaseedharan A (2004) Identification, cloning and sequence analysis of a dwarf genome-specific RAPD marker in rubber [*Hevea brasiliensis* (Muell.) Arg.]. *Plant Cell Rep* 23:327–332
- Wan Abdul Rahaman WY, Ghandimathi H, Othman R, Paranjothy K (1982) Recent developments in tissue culture of *Hevea*. In: Rao AN (ed) Tissue culture of economically important plants. COSTED and Asian Network for Biological Sciences, Singapore, pp 152–158
- Warmke HE (1951) Studies on pollination of *Hevea brasiliensis* in Puerto Rico. *Science* 113:646–648
- Warmke HE (1952) Studies on natural pollination of *Hevea brasiliensis* in Brazil. *Science* 116:474–478

- Wilde HD, Meagher RB, Merkle SA (1992) Expression of foreign genes in transgenic yellow-poplar plants. *Plant Physiol* 98:114–120
- Wititsuwannakul R (1986) Diurnal variation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in latex of *H. brasiliensis* and its relation to rubber content. *Experientia* 42:44–45
- Yeang HY, Chevallier MH (1999) Range of *Hevea brasiliensis* pollen dispersal estimated by esterase isozyme markers. *Ann Bot* 84:681–684
- Yeang HY, Arokiaraj P, Hafsah J, Samsidar H, Siti Arija MA, Jones H (1998) Rubber latex as an expression system for high value proteins. In: Shewry PR, Napier JA, Davies PG (eds) *Engineering crops for industrial end uses*. Portland Press, London, pp 55–64
- Yeang HY, Arokiaraj P, Hafsah J, Siti Arija MA, Rajamanickam S, Chan H, Jafri S, Leelavathy R, Samsidar H, Van der Logt CPE (2002) Expression of a functional recombinant antibody fragment in the latex of transgenic *Hevea brasiliensis*. *J Rubber Res* 5:215–225

## II.3 Eucalyptus

C. TEULIERES and C. MARQUE<sup>1</sup>

### 1 The Importance of Eucalyptus

Native to the Australian continent and its northern neighbors, Eucalyptus is the most widely planted hardwood tree in the world and constitutes one of the world's main sources of biomass. Its versatility and rapid annual growth are still being explored in plantations already estimated at 19 million ha, spread over 37 countries and accounting for 16% of forest plantation areas worldwide (FAO 2000; Carbonnier 2004). India is the largest planter (8 million ha), followed by Brazil (3 million ha) (Junghans et al. 2003), while in Australia and particularly Tasmania, there has also been a move towards growing Eucalyptus in plantations in addition to the 41 million ha of natural Eucalyptus forest (Junghans et al. 2003). Amongst 700 Eucalyptus species, while *E. grandis* is the most widely cultivated species in subtropical and warm temperate regions, *E. camaldulensis* is the most common species in arid and semi-arid lands and *E. globulus* is the main species in temperate climates free of severe frosts. Elite clones are mainly used in Brazil by the cellulose and paper industry because of wood quality and high volume yield. Mature trees also provide strong and durable timber which is the major use of Eucalyptus, along with fuel supply, in countries such as India.

#### 1.1 Requirement for Biotechnological Approaches for Eucalyptus

Increased Eucalyptus plantation productivity and refinements in the quality of wood products as the main parameters of return depend greatly on the use of superior genetic stocks. Most Eucalyptus breeding programs are based on recurrent selection and/or inter-specific hybridization, with domestication involving extensive use of vegetative propagation of individual elite trees. Considering the generation time of Eucalyptus (at least 6 years) and the optimum biological selection age (estimated at 4 years) (Greaves et al. 2003), biotechnology represents a powerful tool to reduce breeding time. This genus, the second target tree for research after pine, accounts for 11% of forestry biotechnology activities and 7% of genetic modification activities (FAO 2004).

---

<sup>1</sup> Pôle de Biotechnologie Végétale, 24, Chemin de Borde Rouge, Auzeville, 31326 Castanet Tolosan, France, e-mail: teulieres@scsv.ups-tlse.fr

## 1.2 What Traits?

The majority of traits of interest to Eucalyptus breeders for genetic improvement through biotechnology concern productivity and wood quality for the paper industry. In addition, stress tolerance and also some more specific traits, such as the synthesis of essential oil, are being investigated for particular pharmaceutical purposes. In order to increase productivity, growth traits such as seedling height or vigor, as well as morphological traits, are being targeted. In addition, with regard to clonal plantations, the capacity of elite Eucalyptus for vegetative propagation, varying within and across Eucalyptus species, is included in breeding programs. Herbicide resistance that could improve viability and growth by facilitating weed control, when young Eucalyptus trees are most vulnerable to competition, is also under investigation. Since most Eucalyptus production goes to the pulp and paper industry, and wood quality requires the tree to attain maturity before assessment, this trait is obviously a major target for biotechnology. Wood density in particular is being studied, as well as lignin quantity and quality which greatly affect cellulose purification.

Biotechnology is being applied to the selection of more frost- or salt-tolerant Eucalyptus to extend plantation areas and to introgress tolerance into the more fast growing and sensitive commercial species. Insect pest and pathogen resistances are also being studied in the larger plantation countries.

## 1.3 What Biotechnological Tools?

In addition to the traditional vegetative propagation and micropropagation still relevant for clonal plantations, biotechnological approaches for Eucalyptus include more sophisticated techniques of genomics, tissue culture and, eventually, gene manipulation. In fact, due probably to a globally poor regeneration ability while exhibiting advantages for genomics approaches through its small genome, Eucalyptus was, with poplar, one of the first forest trees to benefit from the development of new DNA tools. Therefore, and also owing to the side problem of GMOs, research programs on Eucalyptus are less concerned with plant regeneration and transformation and more with transcriptome analysis, molecular markers and mapping. Marker assisted selection (MAS) is expected to be particularly valuable for early selection of Eucalyptus, with regards to their vegetative propagation and physiological characteristics. As for many forest species, MAS could be very useful to help recurrent selection in small elite populations, speeding up recombination and increasing the genetic gain per unit time. Such an approach would make an exceptional contribution to breeding by allowing exploitation of the superior natural phenotypic variation in genetic resources both at the intra- and inter-specific levels. More specifically, it could be used for the selection of individuals at a very juvenile stage before vegetative propagation, thus circumventing the

problem of loss of regeneration and rooting ability, common in *Eucalyptus*. These genomic-based technologies now constitute the first biotechnological tool applied to *Eucalyptus* as confirmed at the international IUFRO meeting, 'Eucalyptus in a Changing World', in Aveiro, Portugal (11–15 October 2004).

#### 1.4 Who is Developing Eucalyptus Biotechnologies?

Most of the *Eucalyptus* genetic engineering technologies are being developed by private companies and almost all the main genomics programs are private or semi-private ventures. Consequently, access to databases is restricted. Therefore, a significant part of the *Eucalyptus* data remains confidential, often patented and only announced in general papers or through poster presentations during conferences. Since very few details are available, this chapter only mentions these important but protected data in passing and focuses more closely on work published in international scientific journals.

First this chapter reviews recent achievements in tissue culture and genetic engineering as applied to *Eucalyptus*. Since a comprehensive review on transgenic *Eucalyptus* was provided by Mac Rae and van Staden (2000), only an update on genetic transformation data is given here. Subsequently, some ongoing genomics research projects are described, in particular with regards to the different consortia that are being initiated to speed up the development of biotechnological tools. The conclusion discusses the main achievements and features of biotechnology as applied to *Eucalyptus*.

## 2 Tissue Culture and Genetic Transformation

Whilst improvement of *Eucalyptus* by biotechnology was, until the 1990s, based mainly on in vitro methods, including genetic engineering, very few papers or reports have been published on the subject recently. It is clear that while some efficient classical techniques, such as micropropagation, are routinely integrated into breeding programs, the design of new regeneration and transformation procedures for novel genotypes is rarely a priority at the present time. Many *Eucalyptus* species are still considered recalcitrant to tissue culture and genetic engineering. In addition, the use of protoplasts and somatic hybridization technology is no longer mentioned for *Eucalyptus* in the literature, probably due to the high recalcitrance of isolated protoplasts. However, 'regenerable' and 'transformable' species, such as *E. grandis* and *E. camaldulensis*, are exploited as models within the genus for testing genes and investigating their function.



## 2.1 Vegetative Propagation

A large proportion of the papers on the subject over the last few years have been provided by South African groups. Most *Eucalyptus* can be propagated vegetatively using traditional stem cutting techniques. However, in vitro micropropagation and rooting, followed by transfer to soil, is becoming increasingly popular because of the prospects of rapid genetic gain. These micropropagated plants are not necessarily used in the establishment of plantations, but are useful source material for the development of clonal hedges and hydroponic systems as sources of cuttings. Micropropagation by microcuttings is commonly carried out on *E. camaldulensis*, *E. globulus*, *E. grandis*, *E. nitens*, *E. tereticornis*, *E. urophylla* and *E. gunnii*. Generally, in vitro plants are produced by the proliferation of shoots from isolated axillary buds. The use of semi-solid systems proved efficient for this multiplication procedure (Watt et al. 2003). For tissue culture, various plant parts have been used as explant material, but the best cultures are generally obtained from juvenile or rejuvenated material; cotyledon or other zygotic embryonal tissues appear to be good sources.

Mondi Forests (P.O. Box 37, Johannesburg 2000, South Africa) has developed conventional micropropagation protocols combining good productivity and low cost for a range of commercially important pure or hybrid *Eucalyptus* genotypes. The explants, selected from seedlings of different provenances, each typically regenerate 10–40 buds and the new shoots are allowed to root in vitro or ex vitro. Both multiplication and rooting rates were found to be greater than with macropropagation. Using this procedure, 30–40 genotypes are routinely multiplied every year, producing 4200 plants per month for field plantation (Bandyopadhyay and Hamill 2000). The field performance of these micropropagated plants was assessed in comparison with macropropagated plants. While physiological characteristics, such as hydraulic conductivity or photosynthetic rates, were not significantly different, growth rate was improved in the plants that originated from micropropagation, allowing a shorter rotation time.

In addition to these conventional multiplication procedures, the production of somatic embryos, which renders micropropagation highly cost effective, would be a powerful tool for the establishment of plantations of elite *Eucalyptus* genotypes. It may be particularly relevant to biotechnology programs involving temperate *Eucalyptus*, such as *E. nitens* and *E. globulus*, because conventional micropropagation procedures involving these species sometimes suffer from the problem of low or variable root formation.

An efficient procedure was designed for plant regeneration of *E. nitens* through somatic embryogenesis from seedling-derived explants (Bandyopadhyay et al. 1999). Strong similarities (size, morphology, cellular organization) were observed between these embryos and zygotic embryos, suggesting very interesting prospects for commercial biotechnology programs based on the production of viable synthetic seeds (Bandyopadhyay and Hamill 2000). In *E. tereticornis*, plants could be produced through embryogenesis, during which a low-light regime at all stages of regeneration is important (Prakash and Gu-

rumurthi 2005). Loureiro et al. (2004) and Oller et al. (2004) also reported the production of somatic embryos in *E. globulus*. Genetic stability of these embryos was assessed by flow cytometry and microsatellites, and the results showed that the protocol used for somatic embryo induction and proliferation did not affect ploidy, DNA content or development conformity of the embryos. In *E. grandis*, plants produced from leaf explants via somatic embryogenesis were evaluated in the field. Interestingly, these plants were found to flower one year earlier than seed-derived plants (Watt et al. 1997). Nevertheless, somatic embryogenesis remains difficult to obtain and has not been reported in a number of Eucalyptus species. These data show that, when mastered on one species, it is a very valuable and efficient tool for Eucalyptus mass propagation.

As a complement to these multiplication strategies, in vitro conservation techniques are required for long-term storage of Eucalyptus useful germplasm and regulation of in vitro production lines according to customer demand. Cryopreservation is a major method used, with good long-term reliability (no exposure to biotic and abiotic stresses) and low cost. It was developed for different Eucalyptus species, such as *E. gunnii* (Paques et al. 1997) and the hybrid *E. grandis* × *E. camaldulensis* (Pendas et al. 2001). In the latter case, the explants consisting of axillary buds were encapsulated in an alginate gel and pre-cultured in media containing a combination of sucrose and glycerol before dehydration and freezing. For isolated *E. grandis* axillary buds, Mycock and colleagues (2004) showed that exposure to abscisic acid (ABA) prior to drying was beneficial in allowing the material to be dried to lower water content without loss of viability or any discernible effect on bud ultra-structure. In addition to this long-term storage using cryopreservation, medium-term storage (up to 6 months) was achieved in Eucalyptus by the maintenance of axillary buds encapsulated in calcium alginate at 10 °C (Watt et al. 2000). Therefore, the main techniques of micropropagation and conservation were developed in the most commercially important Eucalyptus species, with efficiency and reliability compatible to a routine use.

## 2.2 Genetic Modification

Since 2000, reports on the recovery of genetically modified Eucalyptus consist of four research papers in international journals (Harcourt et al. 2000; Chen et al. 2001; Tournier et al. 2003; Valério et al. 2003) and three patents (AU 706 650 B2, EP 1 050 209 A2, US6 255 559 B1) (Cambia 2004). While the research papers describe the production of transgenic Eucalyptus lines carrying a gene of commercial interest, the patents concern only methods of transformation. All these reports have three essential features in common. All the DNA transfers are achieved by using *A. tumefaciens*, the regeneration is always obtained through adventitious organogenesis on tissues, and *E. camaldulensis*, *E. urophylla* or hybrids are always the target species. In addition, and in order to overcome regeneration problems associated with *E. globulus*, Spokevicius et al. (2005)

proposed a new approach based on the introduction of genes into growing wood for analyzing stably transformed wood sectors.

To date, genetic transformation has been used in *Eucalyptus* to modify wood quality (by altering the chemical composition of cell walls in order to impact down-stream processing for desired end-uses), or to increase growth or survival under stress environments (biotic and abiotic). Two complementary strategies have been investigated for improving wood quality. The first one aims to increase cellulose content by introducing two genes (cbd and cel 1, respectively, encoding a cellulose binding domain and a 1-4  $\beta$ -glucanase) into *E. camaldulensis*, *E. grandis* and *E. grandis* hybrids (Shani et al. 2003). Field trials are in progress. The second approach, which is by far the most commonly investigated, aims to decrease lignin content in wood or alter lignin structure to make the lignin easier to extract for the paper industry. An anti-sense construct of Nt lim1, a transcription factor that specifically binds a PAL-box sequence, was introduced into *E. camaldulensis*. The transgenic lines showed decreased expression levels of lignin biosynthesis genes and some of them exhibited reduction of lignin content (Kawaoka et al. 2003). With the same final objective, Chen et al. (2001) transferred the C4H gene (cinnamate 4 hydroxylase), both in sense or anti-sense orientation, into *E. camaldulensis*. Transformation was performed on mature tissues (leaves) from in vitro plantlets derived from elite selected trees. Characterization of two independent lines produced for each construct is in progress. Lastly, a CAD (cinnamyl alcohol dehydrogenase) anti-sense construct was transferred into *E. camaldulensis* (Valério et al. 2003) and the hybrid *E. grandis*  $\times$  *E. urophylla* (Tournier et al. 2003). The CAD gene is considered a good target for modulating lignification, since its downregulation in poplar led to more efficient lignin removal without modifying growth (Baucher et al. 1996). Forty-four individual transgenic lines were produced for *E. camaldulensis*; 32% of them exhibited a significant reduction in CAD activity (up to 83%). Nevertheless, after spending 10 months in the glasshouse, none of the five lines tested showed a significant change in lignin quantity, suggesting that the plants analyzed were not sufficiently suppressed in CAD throughout development. In the case of *E. grandis*  $\times$  *E. urophylla*, 120 transformants were generated, of which 58% were significantly inhibited for CAD activity. Two lines, with respectively 26 and 22% of residual CAD activity, were transferred to the glasshouse for chemical wood analysis. The preliminary data on lignin content without any growth alteration looks promising (unpublished data). More generally, the protocol designed for selected seedlings in this experiment proved efficient enough to generate large populations of transgenic lines compatible with an anti-sense strategy but also with exploitation for gene function studies.

Genetic modification of *Eucalyptus* towards improving growth and viability in abiotic adverse conditions has been mentioned only in posters. In order to improve inorganic phosphate acquisition of *Eucalyptus* on acid soils, a mitochondrial citrate synthase gene was over-expressed in *E. grandis*  $\times$  *E. urophylla* (Kawasu et al. 2003). Enzyme activity was increased up to five-fold compared to

control plants, leading to a similar growth with colloidal aluminium phosphate compared to Na-phosphate. In order to improve adaptation to soil conditions, the *codA* gene from *Arthrobacter globiformis* encoding choline oxidase that converts choline into glycine betain was introduced into Eucalyptus (Yamada-Watanabe et al. 2003). Two transgenic lines more tolerant to high salt stress than wild plants also exhibit a tendency to resist other environmental stresses such as heat and very low temperatures. For more general resistance to environmental stresses, a transcription factor gene (*DREB1*), important for regulating gene expression in response to dehydration, was also over-expressed on Eucalyptus and allowed, in some lines, improved salt tolerance and also drought tolerance (Kondo et al. 2003; Ishige et al. 2004).

Only two articles have been published concerning the use of genetic transformation for increasing tolerance to biotic stress (Harcourt et al. 2000; Shao et al. 2002). Harcourt et al. reported the production of both insect- and herbicide-resistant transgenic *E. camaldulensis*, after transformation of 2-week-old seedlings. These transformants contained both the insecticidal *cry3A* gene and the *bar* gene conferring tolerance to the herbicide ammonium glufosinate. Transgenic plants from two lines tested were resistant to chrysomelid beetles, which are significant pests in Australian commercial Eucalyptus plantations. Shao et al. (2002) reported the insertion of a cecropin D gene into the genome of *E. urophylla* resulting in a 35% increase in resistance to the pathogen *Pseudomonas solanacearum*.

The number of Eucalyptus species subjected to genetic engineering has not progressed in tandem with the number of target genes. Transfer of transformation procedures to more recalcitrant material (adult elite genotypes, other species) has not succeeded. Moreover, the anti-sense strategies used to manipulate wood quality, a major targeted trait in Eucalyptus, showed limited efficiency to significantly decrease lignin content or change its composition. This suggests that other genes remain to be tested (e.g. regulator genes), and more complete gene suppression through procedures such as RNAi type may be required (Wesley et al. 2001). To the best of our knowledge, the application of this technique to Eucalyptus has not been reported.

Genetically modified Eucalyptus was reported in 2004, and 34 field trials were announced worldwide (FAO 2004). However, there was no information regarding gene stability, growth and development, and characteristics of transgenic Eucalyptus lines under field conditions. Although transgene stability after 2 years has already been shown for a reporter gene in *E. camaldulensis* lines (Mac Rae and van Staden 2000), this needs to be confirmed, together with the phenotype for any new gene and any new transgenic line. This long-term analysis is necessary in order to demonstrate fully the value or the unsuitability of gene modulation in terms of genetic improvement.

The future of genetically modified Eucalyptus depends on a demonstration in the field of an agronomic or industrial gain without any negative side effects. It also relies on the absence of environmental risks and the overall acceptability of genetically modified organisms (GMOs). Some factors mitigating the

risks of using transgenics in *Eucalyptus* have been described (Burdon and Walter 2001). Insect pollination of *Eucalyptus*, as opposed to wind pollination, will tend to reduce the risks of long-distance gene flow. Moreover, the very short rotations on which *Eucalyptus* is often grown (7–10 years), usually for pulpwood or fuel, should mitigate the impact of crop failure. In addition, in most countries, apart from Australia, *Eucalyptus* plants are entirely exotic and potential gene flow into natural populations is not an issue. However, in Australia, inter-fertility among *Eucalyptus* species is common, thus favoring cross-pollination among species. The recent upsurge in the establishment of *Eucalyptus* in Australia (Cromer and Eldridge 2000) makes the risk of gene flow a problem on this continent. In Tasmania, hybrids were found at a low frequency as far as 300 m from the parents (Barbour et al. 2002). This problem emphasizes the necessity of obtaining complete sterility in *Eucalyptus*, at least for the Australian plantations. Manipulating the *Eucalyptus* LEAFY gene, functionally equivalent to the *Arabidopsis* floral meristem gene (Rodriguez et al. 2002), could help to achieve this goal. Another alternative suggested by McKinnon et al. (2001) is chloroplast engineering technology, since the *Eucalyptus* chloroplast genome is maternally inherited, at least in the studied species. This strategy, which has now been developed for other plant species, may also allow greater control over gene insertion events. However, it is based on direct transformation procedures which have not proved very efficient in *Eucalyptus*.

Recent data on genetically modified *Eucalyptus* are very promising and deal with an increasing range of genes of commercial interest. However, they also show there is a long way still to go technically in this area. Besides producing commercial GMOs, genetic engineering is dedicated to genomic studies in *Eucalyptus*. Two strategies have proved efficient in this gene analysis. One strategy is, for any trait, the choice of a model genotype adapted to transformation by seedling screening, while the other is, for wood traits, gene transfer into *in vitro* wood formation system.

### 3 *Eucalyptus* Genomics Research Programs

On a worldwide scale, numerous public or private research institutes are focusing their efforts on *Eucalyptus* genomics for species and traits of commercial interest relevant to the geographic zone (Australia, Brazil, Japan, southern Europe and the USA). Structural genomics (linkage mapping and quantitative trait loci detection) and functional studies (construction of cDNA libraries and transcriptome analysis) are the main areas of research. Investigations at the whole genome scale, including systematic sequencing or construction of BAC libraries for physical mapping, are being reported exclusively by the OJI Paper Co. Ltd. (Ginza 4-7-5, Chuo-Ku, Tokyo, Japan) and Genolyptus Brazilian consortium. This section reviews the main goals of *Eucalyptus* genomics

programs, in terms of traits and targeted species, and also emphasizes the technical evolution in the last decade.

### 3.1 Genome Sequencing

Several Eucalyptus genome research programs have been initiated, benefiting from the major technological advances in plant molecular analysis, which have made genome studies much more accessible. These initiatives are motivated by the major economic interest in these species and also by their small genome size (340–580 Mbp), which is 40 times smaller than that of *Pinus* and only 4 times as large as the *Arabidopsis* genome (FAO 2003). Sequencing of the *E. camaldulensis* genome commenced at the beginning of 2004 in the Kasuga DNA Institute in Japan, using public funding and DNA provided by OJI Paper. For this purpose, they produced 112,500 BAC end sequences and 1,072,309 whole genome shotgun sequences (approximately 1X genome equivalent) (A. Myburg, personal communication). In addition, the complete Eucalyptus chloroplast genome has been sequenced by the Cooperative Centre for Sustainable Production Forestry of Tasmania (Steane 2005). This genome was found to be similar to other tree and non-tree angiosperm taxa but different in size, arrangement and gene content from gymnosperms. Moreover, physical mapping is also a very important issue for Eucalyptus, since genetic maps based on recombination frequencies amongst markers have provided a low resolution level on these species. One percent recombination between two markers corresponds, on average, to 0.5 megabase or several dozen genes. The construction of a Eucalyptus BAC library has already been reported for *E. grandis* (Grattapaglia et al. 2004), with a first set of 20,000 clones arrayed from the BAC library (more than 70% of the inserts over 150 kb), giving an estimate of four times coverage (630 Mbp). A second BAC library of *E. globulus* is being constructed in the Brazilian network of Eucalyptus genome research, Genolyptus. Complementing these efforts at the genomic level is gene discovery based on transcriptome analysis.

### 3.2 EST Isolation Programs

Similar to the efforts in poplar, sequencing projects often form part of large genomics programs undertaken by national or international consortia. The biggest on-going joint public and private programs were founded in Brazil, demonstrating the economic importance of Eucalyptus at the state level in this country.

The Genolyptus project (<http://genolyptus.ucb.br/genolyptus-english.jsp>), which includes thirteen companies, seven universities and Embrapa (Empresa Brasileira de Pesquisa Agropecuária), is targeting wood quality and disease resistance. Divided into several topics (Grattapaglia 2004), the research program aims to translate genomics knowledge into improved tree breeding technolo-



gies. In 2004, the sequencing project reached about 150,000 ESTs for *E. grandis* (50%), *E. globulus* (16%), *E. urophylla* (10%) and *E. pellita* (10%), the remaining 14% for six other species. To date, access to the sequence database is restricted to the consortium. The second Brazilian consortium, FOREST (<http://est.cbmeg.unicamp.br/pgl/research/forests.html>), is made up of twenty laboratories from San Paulo and four companies. The isolation of 124,000 sequences (12 cDNA libraries) was obtained mainly from *E. grandis*, representing different organs under different growth conditions and related to environmental stress tolerance (Furtado et al. 2004). In addition, OJI Paper has 60,000 ESTs.

Another independent French public genome sequencing program (26,000 ESTs from *E. gunnii*) was also named FOREST (<http://www.genoscope.cns.fr>). These Eucalyptus sequences were isolated in the framework of the Toulouse University-CNRS research program, focusing on the regulation of lignin biosynthesis ([http://www.smcv.ups-tlse.fr/root/equipes/regulation/equipe\\_en.php](http://www.smcv.ups-tlse.fr/root/equipes/regulation/equipe_en.php)) and frost tolerance ([http://www.smcv.ups-tlse.fr/root/equipes/stressfroid/equipe\\_en.php](http://www.smcv.ups-tlse.fr/root/equipes/stressfroid/equipe_en.php)). The sequences are being released on GenBank.

In Australia, the CSIRO forestry research programs (<http://www.ffp.csiro.au/tigr/molecular/>) led to the isolation of EST collections from *E. grandis* and *E. grandis* × *E. nitens* hybrids. A current research project on wood quality is based on a 5000 cDNA library from Eucalyptus xylem (Moran et al. 2002). Moreover, the same research groups are also interested in flowering (Watson and Brill 2004) and cold tolerance (Fullard and Moran 2003). At the University of Melbourne, 93 sequences of cambial genes from *E. globulus* have been posted in GenBank (accession nos AW191301–AW191393) and, from them, 43 could be annotated (Bossinger and Leitch 2000).

In the Forest Biotechnology group of the NCSU (North Carolina State University), 555 ESTs were generated from differentiating xylem of a hybrid *E. grandis* × *E. globulus* and are available on GenBank (accession nos CB967505–CB968059). In conjunction with the Dupont de Nemours Company, the same group developed a Eucalyptus EST project resulting in 2150 sequences isolated from different organs of *E. grandis* and *E. tereticornis*, and these sequences were deposited in GenBank (accession nos CD667988–CD670137).

Finally, combining ArborGen and Genesis (in the USA and New Zealand, respectively), Agrigenesis (<http://www.agrigenesis.com>) is a new private company involved in Eucalyptus EST sequencing. A total of 218,000 sequences (23,000 contigs) were isolated from different tissues (xylem or phloem or photosynthetic tissues), different organs (roots or reproductive organs), developing embryos and seedlings of various species (*E. gunnii*, *E. dunnii*, *E. grandis* and *E. obliqua*). From this collection, 951 have been put in GenBank (Strabala 2004).

To summarize, different sets of ESTs (a global estimation is about 500,000 sequences) have already been produced, mainly on *E. grandis* and concerning, in particular, wood quality. These EST collections are useful for microarray design, annotation of the genome sequence and the development of molecular markers important in anchoring the genome contig sequences.



### 3.3 Gene Annotation and Global Expression Analysis

Through DNA-array technology, global analyses provide the opportunity to study gene expression patterns, gene interactions and co-regulation. Analyses were applied in particular to the study of wood formation by analysing cDNA subtractive libraries enriched in cambium genes (Bossinger and Leitch 2000) or xylem genes (Paux et al. 2004; Qiu et al. 2005). The first study yielded a number of novel cambial cDNA fragments, some of which exhibited a strong cambium-specific expression. To directly isolate candidate genes associated with growth, the NCSU has developed an innovative global strategy associating the transcript abundance of 2608 genes with the phenotypic variation (diameter growth) of the progeny of an elite hybrid of *E. globulus* × *E. grandis* (Kirst et al. 2004b). The results revealed a coordinated reduction in transcript level for genes encoding enzymes involved in lignin biosynthesis in the hybrid progeny that displayed superior growth. Lignin analysis of wood samples confirmed the content and quality predicted by gene profiling data. Quantitative trait loci (QTL) analysis of gene expression indicated that eQTLs (e for expression) of lignin-related genes co-localized with growth QTLs, which is evidence of common regulation. According to Paux et al. (2004), the expression results, confirmed by real-time RT-PCR, showed that 81% of 224 ESTs were preferentially expressed in differentiating secondary xylem.

Apart from wood formation and wood quality, investigations are also being conducted in the field of abiotic stresses. Using a suppressive subtractive hybridization (SSH), two cDNA libraries (forward and reverse SSH), were constructed in order to isolate a pool of genes regulated during cold acclimation (El Kayal 2004). The DNA-array results, validated by Northern analysis, showed a general pattern of gene co-regulation by cold and osmotic stress. In addition, a complete cDNA library was produced from cold-acclimated *E. gunnii* leaves. Among the 366,000 ESTs, 13,500 were sequenced and 3840 analysed for gene identity and expression (Keller 2006). Besides the known genes, 38% of sequences were found without any homology in the databases (recorded as 'no hit') similarly to other data on *Eucalyptus* (Paux et al. 2004). In the cold-acclimation library, numerous genes are involved in cell rescue and gene transcription as well as sugar and lipid metabolism. In the study of xylogenesis in *E. gunnii*, one third of the sequences are related to known processes of cell signalling and cell wall biogenesis, and some of the remaining sequences are under investigation to provide new insights into xylogenesis (Paux et al. 2004). It was shown that 415 tags from 11,045 ESTs of young *E. grandis* stems were highly expressed. The functional category containing genes involved in energy production and cellular maintenance was most represented, followed by growth, development and cellular regulation controlling genes (Carneiro et al. 2005). In *E. nitens*, genes potentially involved in the control of wood fiber properties, such as a cellulose synthase gene, were identified (Qiu et al. 2005).

More importantly, these studies allowed the isolation of important genes encoding transcription factors such as EgMYB2, regulating secondary cell wall

formation (Goicoechea et al. 2005), or EguCBF1 a and b, known for their key role in cold acclimation (El Kayal et al. 2006a). Quantitative expression analysis revealed interesting features of these Eucalyptus genes compared with the data from the literature concerning mainly herbaceous plants. The results on Eucalyptus have revealed new biological roles for genes with known functions; for example, for the first time the EgSxd1 gene, encoding the tocopherol cyclase (VitE), was found to be regulated by abiotic stress (El Kayal et al. 2006b).

The results of gene profiling are very promising although not very well documented because of the novelty of gene discovery projects. In the near future, the accumulation of transcriptome analysis data should allow understanding of the molecular basis of Eucalyptus specificity and the identification of candidate genes for MAS.

### 3.4 Analysis of the Genetic Control of Complex Quantitative Traits and Mapping

Genetic maps are powerful tools to assist breeding for Eucalyptus improvement. The different molecular markers used for mapping are strongly dependent on DNA-technology advances, and have moved recently from anonymous markers to those associated with candidate genes. Among the targeted species, *E. globulus* appears to be studied all around the world, probably due to its exceptional wood quality, combined with fast growth. *E. grandis* and *E. urophylla* as highly productive species and *E. nitens* as cold-adaptable are also well investigated. Wood quality is the main trait studied for all the Eucalyptus species. Depending on the species and the geographic location, other traits are more specific. The latter include growth, disease resistance and frost or drought tolerance, flowering, and rooting ability for vegetative propagation.

During the last decade, a number of genetic maps based on DNA markers have been constructed for individual pedigrees of the main Eucalyptus commercial species. Historically, a map based on RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphism DNA) markers was developed in *E. nitens* (Byrne et al. 1995) and an RAPD map in *E. grandis* × *E. urophylla* (Grattapaglia and Sederoff 1994). More recently, largely dominant markers such as AFLPs (amplified fragment length polymorphism) and RAPDs were used for map construction from hybrid crosses of *E. grandis* and *E. globulus* (Marques et al. 1998; Myburg et al. 2003). In addition, the microsatellite markers (SSRs, single sequence repeats) have been developed in order to complete linkage maps (Brondani et al. 2002). The SSR markers were associated with RFLPs, candidate genes (CG) and isozymes for linkage map construction in *E. globulus* (Thamarus et al. 2002). Despite their high development cost, SSR markers remain very commonly used. Multi-allelic and co-dominant, they are known to be highly informative and very powerful for understanding the allelic variation segregating at QTLs. Besides being a suitable tool in quantitative trait analysis and marker assisted breeding,

microsatellites are also valuable for screening large insert libraries, prior to cloning (Chen et al. 1997). A database for cataloging Eucalyptus EST-derived SSRs was created by the FOREST consortium, and a bioinformatics tool named Satellyptus was developed for searching and analyzing microsatellites in the Eucalyptus EST database (Ceresini et al. 2005). The first data show that most of the microsatellites detected in the Eucalyptus ESTs are positioned at either the 3' or 5' end. The Genolyptus program is still developing a new set of SSR markers from BAC or EST libraries, which could provide an immediate mapping of genes and, potentially, the suggestion of candidate loci co-localizing with QTLs (Grattapaglia et al. 2004). To obtain a general overview of the structure and composition of the *E. grandis* genome, this consortium sequenced 10,000 randomly sheared genomic DNA clones and identified 987 microsatellites, with a frequency of one perfect microsatellite every 12 Kbp in the genome (Lourenço et al. 2005). Generally assayable across the Eucalyptus species (Moran et al. 2002), the SSR markers, along with candidate genes, proved to be also useful in intraspecific genetic research and integration of maps across species, but have been less utilized in phylogenetic reconstruction (Marques et al. 2002).

The constructed maps have provided sufficient genome coverage for the identification of QTLs that have a significant effect on the expression of important traits (Brondani et al. 2002). Thamarus et al. (2004) identified five new QTLs for wood density, pulp yield and microfibril angle. Currently, the quality of these QTLs in Eucalyptus is being improved through the use of SSR markers, much larger sample sizes and new pedigree structures allowing better QTL detection, more accurate estimation of magnitude of effect and more efficient validation. Although several maps of Eucalyptus have been constructed, the possibility of sharing QTL data was restricted by the low transferability of the dominant markers, but the development of SSR should now allow information exchange across species of the same genus. For example, among four Eucalyptus species, Marques et al. (2002) demonstrated the conservation and the syntheny of SSRs and QTLs for vegetative propagation, which are located on homeologous linkage groups. Using the same approach, Brondani et al. (2002) developed a reference map for the genus Eucalyptus based on microsatellite markers and RAPD framework linkage maps of *E. grandis* and *E. urophylla*, indicating successful integration between data obtained using different techniques. These results will facilitate investigation of new allelic variations within and among species, giving more opportunity for MAS (Junghans et al. 2003).

Identification of the major genes underlying these quantitative traits is one of the main aims of genomics in order to provide reliable markers. From an operational point of view, the CG approach has the advantage that the selection could be practised directly on the gene and therefore would not rely on the need for strong association linkage disequilibrium (LD) between the marker allele and the favorable allele at the gene of interest. These candidate genes have been isolated through protein purification and orthologous gene screening and, more recently, through EST isolation. New potential candidate genes may be chosen according to their function or from differential expression

patterns from global analysis. Association studies are then necessary to relate a DNA-polymorphism to a phenotypic variation for the corresponding traits. At the nucleotide level, the SNPs (single nucleotide polymorphisms) are the most precise markers that could be used for these studies. This candidate gene approach was extensively evaluated for the identification of selection markers for wood quality, but, to date, only a few genes of interest have been localized on *Eucalyptus* maps.

The amount of data concerning wood formation in *Eucalyptus* generated by various international groups provides a good illustration of candidate gene strategy. First, genes involved in lignin synthesis, such as CAD (cinnamyl alcohol dehydrogenase) and CCR (cinnamoyl CoA reductase), were isolated after protein purification from xylem tissues (Goffner et al. 1992, 1994). In addition to this targeted 'a priori' process, recently a global approach has allowed the identification of dozens of genes preferentially expressed in xylem (Bossinger and Leitch 2000; Moran et al. 2002; Kirst et al. 2004b; Paux et al. 2004). The positive impact of repressing gene expression on lignin content and/or composition was demonstrated for several genes in model plants (tobacco and poplar) and evaluated in *Eucalyptus* (Tournier et al. 2003; Valério et al. 2003).

CAD and CCR, as well as four other genes involved in the common phenylpropanoid pathway, were found to be located in four distinct regions of the *E. urophylla* RAPD map (Gion et al. 2000), and the CCR gene did not co-localize with known wood quality QTLs (Poke et al. 2004). Fifty-three SNPs were identified for the CCR sequence and 8 for CAD on *E. globulus*, but up till now, association studies have not shown any evidence that these single amino-acid substitutions in CCR affect lignin content (Poke et al. 2003). In the same species, 25 SNPs on coding or non-coding CAD sequences were reported independently, indicating high nucleotide diversity for this gene (Kirst et al. 2004a). Association studies with wood traits are in progress.

Altogether, these data emphasize the significant advances in *Eucalyptus* genomics concerning wood quality, as a result of a huge research effort justified by the major foreseeable impact of MAS for this trait. This progress was facilitated by the emergence of both molecular technologies and precise wood and fiber assessment methods necessary for the phenotyping. Obviously, *E. globulus* germplasm stands out as a very rich source of genetic variation for all the target wood traits and, therefore, represents a key genetic resource for these studies. The number and, above all, the quality of QTLs has increased and, in the last decade, the second generation of markers, used for the construction of genetic maps, has generated a sound foundation to establish MAS. The latter is moving from anonymous markers towards candidate gene strategy, more precise and reliable. This orientation, already initiated by different groups in *Eucalyptus*, is made possible by the identification of numerous potential candidate genes, thanks to the advances in physical mapping and EST discovery, combined with DNA-array technology. A number of genes, mainly involved in lignin and cell wall biosynthesis, are being validated and appear as good potential candidates. In the near future, this approach will also concern regulatory

genes such as transcription factors, likely to have a high impact on the trait. The first data on Eucalyptus candidate genes so far indicate that major effect genes exist, suggesting that a 'gene-assisted technology' is a realistic strategy for the implementation of MAS.

## 4 Conclusions and Perspectives

Due to its very high economic importance, Eucalyptus was one of the first trees after poplar to benefit from new biotechnological developments. However, application of these tools is confronted, first, with the problem of the globally low regenerative ability of the genus, restricting the availability of genetic transformation procedures to a very limited range of species and genotypes. Second, access to most of the protocols and data, including genetic engineering and field trial results as well as EST sequence databases, is severely limited by their confidentiality due to the commercial interest of these tools or products. This situation is hindering the application of biotechnologies to Eucalyptus and makes it difficult to give a precise state-of-the-art.

The use of seedling material appears to be the most realistic option at present in order to overcome the first problem and produce genetically modified Eucalyptus combining a new trait with a superior genetic background. This genetic transformation strategy consists of selecting, amongst a seed lot from superior parents, young seedlings exhibiting very good regeneration and transformation ability. Downstream, this approach still requires evaluation of the transgenic lines for growth and industrial properties. However, in the future, the availability of molecular markers for these characteristics should allow an early evaluation of the seedlings before introducing the target gene. This would be an interesting achievement of MAS in the field of genetic engineering, and would provide new stimulation to the genetic transformation of superior genotypes.

In terms of the secrecy surrounding a large part of the projects and data on Eucalyptus, fortunately it does not concern the whole genome sequencing project. Moreover, since 2001 it has been slightly aided by initiatives creating formalized consortia such as Genolyptus and Forest, or simply exchanging data or tools. In the area of genetic engineering, although genetic transformation procedures associated with elite genotypes are likely to remain industrial properties, the Eucalyptus model systems adapted for functional evaluation of genes may become more publicly available.

In the area of genomics, many results have already been published, in particular mapping data, including results from programs involving public and private groups, such as the Brazilian ones. Unfortunately, the majority of Eucalyptus sequences (genomic sequences and ESTs) are not available in the public databases, thereby slowing down the general effort of gene identification. However, thanks to general knowledge about plant genomics (poplar and

Arabidopsis) and to technological progress, such a task is made easier and has allowed most of the groups (consortia or individuals) to build their own databases. Even more promising, there is an international initiative on Eucalyptus genomics, with similar features to the 'International Poplar Genome Consortium', and with the final goal of establishing a series of public resources for Eucalyptus genomics research. This initiative, the International Eucalyptus Genome Consortium led by A. Myburg (South Africa), was named EUCAGEN; <http://www.ieugc.up.ac.za>, and has been submitted to the US Department of Energy (DOE) Joint Genome Institute (IGI). The DOE has short-listed the sequencing of *E. grandis* in 2007 (4X coverage) using the shotgun approach. Most importantly, this project will render the DNA sequences completely public and allow the integration of these sequences with both genetic and physical mapping resources provided by various groups from Brazil, Australia, South Africa, Europe and the USA.

In summary, the framework of biotechnologies as applied to Eucalyptus has been constructed largely in the last 10 years. It remains very dynamic, because of major research efforts on a worldwide scale, and is increasingly orientated toward genomics and less and less focused on tissue culture and genetic engineering aspects. There is also a significant change in the organization of this research, with the initiation of consortia and the multiplication of collaborative projects, and, finally, the promise of an international consortium.

**Acknowledgements.** Thanks to Gavin Moran for critical reading of our manuscript and useful comments.

## References

- Bandyopadhyay S, Hamill JD (2000) Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. *Ann Bot* 86:237–244
- Bandyopadhyay S, Cane K, Rasmussen G, Hamill JD (1999) Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species *Eucalyptus nitens* and *E. globulus*. *Plant Sci* 140:189–198
- Barbour R, Potts B, Vaillancourt R, Tibbits W, Wiltshire R (2002) Gene flow between introduced and native *Eucalyptus* species. *New For* 23:177–191
- Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, Cornu D, Monties B, Van Montagu M, Inze D, Jouanin L, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. *Plant Physiol* 112:1479–1490
- Bossinger G, Leitch M (2000) Isolation of cambium-specific genes from *Eucalyptus globulus* Labill. In: Savidge R, Barnett J, Napier R (eds) *Cell and molecular biology of wood formation*. BIOS Scientific, Oxford, pp 203–207
- Brondani RP, Brondani C, Grattapaglia D (2002) Towards a genus-wide reference linkage map for *Eucalyptus* based exclusively on highly informative microsatellite markers. *Mol Gen Genet* 267:338–347
- Burdon R, Walter C (2001) Perspectives on risk in transgenic forest plantations in relation to conventional breeding and use of exotic pines and eucalypts: viewpoints of practicing breeding and transformation scientists. In: Strauss S, Bradshaw H (eds) *Proc 1st Int Symp on*



- Ecological and Societal Aspects of Transgenic Plantations, College of Forestry, Oregon State University, Stevenson, pp 124–137
- Byrne M, Murrell J, Allen B, Moran GF (1995) An integrated genetic linkage map for eucalypts using RFLP, RAPD and isozyme markers. *Theor Appl Genet* 91:869–875
- Cambia (2004) Eucalyptus transformation. <http://cambia.cambia.org.au/cgi-bin/cipr/tt3-bios/simple.cgi>
- Carbonnier L (2004) The future of *Eucalyptus* plantations. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO on silviculture and improvement of eucalypts: Eucalyptus in a changing world. Raiz Instituto, Aveiro, p 29
- Carneiro R, De Carvalho M, Gomes D, Salvatierra G, Moon D, Oda S, Labate C (2005) Serial analysis of gene expression (SAGE) in young *E. grandis* plant stems. In: Wingfield BD, Myburg A (eds) IUFRO tree biotechnology. University of Pretoria, Pretoria, p S1.11
- Ceresini P, Silva C, Missio R, Souza E, Fischer C, Guilherme I, Gregorio I, Da Silva E, Cicarelli R, Da Silva M, Garcia J, Avelar G, Porto Neto L, AR M, Junior M, Marini D (2005) Satellite: analysis and databases of microsatellites from ESTs of Eucalyptus. *Genet Mol Biol* 28:589–600
- Chen X, Temnykh S, Xu Y, Cho Y, McCouch S (1997) Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). *Theor Appl Genet* 95:553–567
- Chen Z, Chang S, Ho C, Chen Y, Tsai J, Chiang V (2001) Plant production of transgenic *Eucalyptus camaldulensis* carrying the *Populus tremuloides* cinnamate 4-hydroxylase gene. *Taiwan J For Sci* 16:249–258
- Cromer RN, Eldridge KG (2000) The Eucalypts as tree crops. In: Last FT (ed) Ecosystems of the world, tree crop ecosystems. Elsevier, Amsterdam, pp 226–269
- El Kayal W (2004) Réponse aux stress abiotiques chez *Eucalyptus gunnii*: analyse globale du transcriptome sur filters haute-densité et caractérisation du gene Sxdl (synthèse de la vitamine E). UMR 5546, Université Paul Sabatier, Toulouse, p 110
- El Kayal W, Navarro M, Marque G, Keller G, Marque C, Teulieres C (2006a) Expression profile of CBF-like transcriptional factor genes from Eucalyptus in response to cold. *J Exp Bot* 57:2455–2469
- El Kayal W, Keller G, Debayles C, Kumar R, Weier D, Teulieres C, Marque C (2006b) Regulation of tocopherol biosynthesis through transcriptional control of tocopherol cyclase during cold hardening in *Eucalyptus gunnii*. *Physiol Plant* 126:212–223
- FAO (2000) Global forest resource assessment. Main report. Forestry Paper 140. FAO, Rome
- FAO (2003) How forest genetic conservation can benefit from new achievements in genomics. <http://www.fao.org/docrep/003/x6884e/x6884e03.htm>
- FAO (2004) Preliminary review of biotechnology in forestry, including genetic modification. Forest Genetic Resources Working Papers FGR/59E. Forest Resources Development Service, Forest Resources Division. FAO, Rome
- Fullard K, Moran G (2003) Identification of frost tolerance genes in a Eucalyptus hybrid cross. In: Sundberg B (ed) IUFRO tree biotechnology. Umea Plant Science Centre, Umea, pp S6–S18
- Furtado E, Rosa D, Oliveira R, Velini E, Alves E, Mori E, Guerrini I, Wincken C, Maia I, Marino C (2004) Mining of *Eucalyptus* ESTs involved in the mechanisms against plant pathogen and environmental stress. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, pp 364–372
- Gion J, Rech P, Grima-Pettenati J, Verhaegen D, Plomion C (2000) Mapping candidate genes in *Eucalyptus* with emphasis on lignification genes. *Mol Breed* 6:441–449
- Goffner D, Joffroy I, Grima-Pettenati J, Halpin C, Knight M, Schuch W, Boudet AM (1992) Purification and characterization of isoforms of cinnamyl alcohol dehydrogenase (CAD) from *Eucalyptus* xylem. *Planta* 188:48–53
- Goffner D, Campbell M, Campargue C, Clastre M, Borderies G, Boudet A, Boudet AM (1994) Purification and characterization of cinnamoyl-coenzyme A: NADP oxidoreductase in *Eucalyptus gunnii*. *Plant Physiol* 106:625–632
- Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati J (2005) EgMYB2, a new transcriptional activator



- from *Eucalyptus* xylem, regulates secondary cell wall formation and lignin biosynthesis. *Plant J* 43:553–567
- Grattapaglia D (2004) Integrating genomics into *Eucalyptus* breeding. *Genet Mol Res* 3:369–379
- Grattapaglia D, Sederoff RR (1994) Genetic linkage map of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross mapping strategy and RAPD markers. *Genetics* 137:1121–1137
- Grattapaglia D, Alfenas A, Coelho A, Bearzoti E, Pappas G, Pascali G, Pereira G, Colodette J, Gomide J, Bueno J, Cascardo JC, Brondani R, Brommonschenkel S (2004) Building resources for molecular breeding of *Eucalyptus*: the Genolyptus project in Brazil. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, pp 30–36
- Greaves BL, Borralho NMG, Raymond CA (2003) Early selection in eucalypt breeding in Australia – optimum selection age to minimise the total cost of kraft pulp production. *New For* 25:201–210
- Harcourt R, Kyoizuka J, Floyd RB, Bateman KS, Tanaka H, Decroocq V, Llewellyn DJ, Zhu X, Peacock WJ, Dennis ES (2000) Insect- and herbicide-resistant transgenic eucalypts. *Mol Breed* 6:307–315
- Ishige N, Kondo K, Furujyo A, Hibino T (2004) Genetic improvement for environmental stress resistance in *Eucalyptus*. In: *Proc Plant and Animal Genome 12th Conf*, San Diego, California, 10–14 January, <http://www.intl-pag.org/12/abstracts>
- Junghans D, Alfenas A, Brommonschenkel S, Oda S, Mello E, Grattapaglia D (2003) Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. *Theor Appl Genet* 108:175–180
- Kawaoka A, Nanto K, Sugita K, Endo S, Yamada-Watanabe K, Matsunaga E, Ebinuma H (2003) Production and analysis of lignin-modified transgenic *Eucalyptus*. In: Sundberg B (ed) IUFRO tree biotechnology. Uméa Plant Science Centre, Uméa, pp S10–S16
- Kawasu T, Susuki Y, Wada T, Kondo K, Koyama H (2003) Overexpression of a plant mitochondrial citrate synthase in *Eucalyptus* trees improved growth when cultured by Alphosphate as a sole phosphate source. *Plant Cell Physiol* 44:S91
- Keller G (2006) Analyse du transcriptome de l'*Eucalyptus* pendant l'acclimatation au froid; recherche de gènes candidats de la tolérance au gel. PhD thesis, Paul Sabatier University, Toulouse
- Kirst M, Marques C, Sederoff R (2004a) SNP discovery, diversity and association studies in *Eucalyptus*. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, p 412
- Kirst M, Myburg AA, De Leon JP, Kirst ME, Scott J, Sederoff R (2004b) Coordinated genetic regulation of growth and lignin revealed by quantitative trait locus analysis of cDNA microarray data in an interspecific backcross of *Eucalyptus*. *Plant Physiol* 135:2368–2378
- Kondo K, Furujyo A, Ishige N, Kasuga M, Shinozaki K, Yamaguchi-Shinozaki K, Hibino T (2003) Analysis of the stress response genes in *Eucalyptus* and effect of introducing several stress tolerance-giving genes into *Eucalyptus*; a development situation and a practical possibility of an environmental stress resistant tree. In: *Proc Plant and Animal Genome 11th Conf*, San Diego, California, [http://www.intl-pag.org/11/p7b\\_p826\\_xi.html](http://www.intl-pag.org/11/p7b_p826_xi.html)
- Loureiro J, Lopez T, Pinto G, Santos R, Silva S, Santos C (2004) Analysis of genetic stability of *Eucalyptus globulus* Labill. Somatic embryos by flow cytometry and microsatellites. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, p 529
- Lourenço R, Grattapaglia D, Pappas Jr G, Pereira G (2005) Sample sequencing of three megabases of shotgun DNA of *E. grandis*: genome structure, repetitive elements and genes. In: Wingfield BD, Myburg A (eds) IUFRO tree biotechnology. University of Pretoria, Pretoria, p S1.8
- Mac Rae S, van Staden J (2000) Transgenic *Eucalyptus*. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 44. Transgenic trees. Springer, Berlin Heidelberg New York, pp 88–112

- Marques C, Araujo J, Ferreira J, Whetten R, O'Malley D, Liu B, Sederoff R (1998) AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. *Theor Appl Genet* 96:727–737
- Marques C, Brondani R, Grattapaglia D, Sederoff R (2002) Conservation and syntheny of SSR loci and QTLs for vegetative propagation in four *Eucalyptus* species. *Theor Appl Genet* 105:474–478
- McKinnon AE, Vaillancourt RE, Tilyard PA, Potts BM (2001) Maternal inheritance of the chloroplast genome in *Eucalyptus globulus* and interspecific hybrids. *Genome* 44:831–835
- Moran GF, Thamarus K, Raymond CA, Qiu D, Uren T, Southerton S (2002) Genomics of eucalyptus wood traits. *Ann For Sci* 59:645–650
- Myburg AA, Griffin AR, Sederoff RR, Whetten RW (2003) Comparative genetic linkage maps of *Eucalyptus grandis*, *Eucalyptus globulus* and their F1 hybrid based on a double pseudo-backcross mapping approach. *Theor Appl Genet* 107:1028–1042
- Mycock D, Mlambo C, Padayachee K, Blakeway F, Watt M (2004) The role of in vitro storage technology in the maintenance of *Eucalyptus* germplasm. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, pp 534–535
- Oller J, Toribio M, Celestino C, Tobal G (2004) The culture of elite adult trees in a genetic improvement programme through *Eucalyptus globulus* Labill. clonal micropropagation. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO on silviculture and improvement of eucalypts: *Eucalyptus* in a changing world. Raiz Instituto, Aveiro, p 539
- Paques M, Poissonnier M, Dumas E, Monod V (1997) Cryopreservation of dormant and non dormant broad-leaved trees. *Acta Hort* 447:491–497
- Paux E, Tamasloukht M, Ladouce N, Sivadon P, Grima-Pettenati J (2004) Identification of genes preferentially expressed during wood formation in *Eucalyptus*. *Plant Mol Biol* 55:263–280
- Pendas J, Moreira T, Guerra O, Pena BR, Fernandez JA (2001) Water relationships in *Phyllanthus orbicularis* and *Punica granatum* antiviral extracts and their influence on stability after freezing and freeze-drying. *Cryo Lett* 22:5–12
- Poke F, Vaillancourt RE, Elliott R, Reid J (2003) Sequence variation in two lignin biosynthesis genes, cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase 2 (CAD2). *Mol Breed* 12:107–118
- Poke F, Raymond CA, Reid J, Vaillancourt RE (2004) The effect of a single amino acid substitution in a lignin biosynthesis enzyme on wood properties in *Eucalyptus globulus*. In: Borralho N, Pereira J, Marques C, Coutinho J, Madeira M, Tomé M (eds) IUFRO silviculture and improvement of eucalypts. Raiz Instituto, Aveiro, pp 388–394
- Prakash M, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *E. tereticornis* Sm. *Curr Sci* 88:1311–1316
- Qiu D, Wilson IRW, Moran GF, Southerton S (2005) Identification of genes controlling wood fibre properties in *E. nitens*. In: Wingfield DB, Myburg A (eds) IUFRO tree biotechnology. University of Pretoria, Pretoria, p S1.3
- Rodriguez A, Mendes da Gloria F, Dornelas M, Amaral W (2002) Towards obtaining an early flowering *Eucalyptus*. In: Proc American Society of Plant Biologists Meeting, Denver, Colorado
- Shani Z, Dekel M, Cohen B, Barimboim N, Kolosovski N, Safranuvitch A, Cohen O, Shoseyov O (2003) Cell wall modification for the enhancement of commercial *Eucalyptus* species. In: Sundberg B (ed) IUFRO tree biotechnology. Uméa Plant Science Centre, Uméa, pp S10–S26
- Shao Z, Chen W, Luo H, Ye X, Zhan J (2002) Studies on the introduction of the cecropin D gene into *Eucalyptus urophylla* to breed the resistant varieties to *Pseudomonas solanacearum*. *Sci Silvae Sin* 38:92–97
- Spokevicius AV, Van Beveren K, Leitch M, Bossinger G (2005) *Agrobacterium*-mediated in vitro transformation of wood-producing stem segments in eucalypts. *Plant Cell Rep* 23:617–624
- Steane DA (2005) Complete nucleotide sequence of the chloroplast genome from the Tasmanian Blue Gum, *Eucalyptus globulus* (Myrtaceae). *DNA Res* 12:215–220
- Strabala T (2004) Expressed sequence tag databases from forestry tree species. In: Kumar S, Fladung M (eds) Molecular genetics and breeding of forest trees. Haworth Press, New York, pp 19–52

- Thamarus KA, Groom K, Murrell J, Byrne M, Moran GF (2002) A genetic linkage map for *Eucalyptus globulus* with candidate loci for wood, fibre, and floral traits. *Theor Appl Genet* 104:379–387
- Thamarus K, Groom K, Bradley A, Raymond CA, Schimleck LR, Williams ER, Moran GF (2004) Identification of quantitative trait loci for wood and fibre properties in two full-sib properties of *Eucalyptus globulus*. *Theor Appl Genet* 109:856–864
- Tournier V, Grat S, Marque C, El Kayal W, Penchel R, de Andrade G, Boudet A-M, Teulieres C (2003) An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* × *Eucalyptus urophylla*). *Transgenic Res* 12:403–411
- Valério L, Carter D, Rodrigues JC, Tournier V, Gominho J, Marque C, Boudet A-M, Maunders M, Pereira H, Teulieres C (2003) Down regulation of cinnamyl alcohol dehydrogenase, a lignification enzyme, in *Eucalyptus camaldulensis*. *Mol Breed* 12:157–167
- Watson J, Brill E (2004) *Eucalyptus grandis* has at least two functional *SOC1*-like floral activator genes. *Func Plant Biol* 31:225–234
- Watt M, Blakeway F, Herman B, Denison M (1997) Biotechnologie et programme d'amélioration génétique dans la foresterie commerciale en Afrique du Sud. In: FAO (eds) *Proc 11th Congr Forestier Mondial*, Antalya, pp 197–203
- Watt M, Thokoane N, Mycock D, Blakeway F (2000) In vitro storage of *Eucalyptus grandis* germplasm under minimum growth conditions. *Plant Cell Tissue Organ Cult* 61:161–164
- Watt M, Blakeway F, Mokotedi M, Jain S (2003) Micropropagation of *Eucalyptus*. In: Jain S, Ischii K (eds) *Micropropagation of woody trees and fruits*. Kluwer, Dordrecht, pp 217–244
- Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27:581–590
- Yamada-Watanabe K, Kawaoka A, Matsunaga K, Nanto K, Sugita K, Endo S, Ebinuma H, Murata N (2003) Molecular breeding of *Eucalyptus*: analysis of salt stress tolerance of transgenic *Eucalyptus camaldulensis* that over-expressed choline oxidase gene (*codA*). In: Sundberg B (ed) *IUFRO tree biotechnology*. Uméa Plant Science Centre, Uméa, pp S7–S9

## II.4 Tropical Tree Legumes

Y. HONG and S. BHATNAGAR<sup>1</sup>

### 1 Introduction

Legumes are members of the family Fabaceae, a diverse group of bean-pod bearing plants ranging from small annual herbs, through woody shrubs to giant perennial trees. Fabaceae is the third largest flowering plant family after orchids (Orchidaceae) and asters (Asteraceae), comprising around 700 genera and 20,000 species (Doyle and Luckow 2003). Fabaceae grow in a variety of soil conditions and growth habitats throughout the world, but predominantly in arid and also rain-fed tropical zones. The family is divided into the subfamilies Papilionoideae, Caesalpinioideae and Mimosoideae based on floral and other morphological characters. The Papilionoideae is the largest subfamily, with 476 genera and about 14,000 species (Lewis et al. 2003) and characteristic papilionaceous (butterfly-like) flowers (five petals are differentiated into two standards, two wings and one keel). This group of plants is mostly herbaceous, and includes cultivated crop legumes such as peanut (*Arachis hypogaea*), soybean (*Glycine max*), lentil (*Lens culinaris*), barrel medic (*Medicago truncatula*), French bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*), which are rich protein sources in human and animal diets. The majority of the Caesalpinioideae and Mimosoideae are tropical or subtropical trees and shrubs. The Caesalpinioideae has irregular but less differentiated flowers (zygomorphic), with stamens visible externally. Tree species in this subfamily include tropical ornamentals (e.g. the flamboyant tree, *Delonix regia*), medicinal plants (e.g. Alexandrian senna, *Senna alexandrina*), fruit trees (e.g. tamarind, *Tamarindus indica*), and those used for timber (e.g. Indian rosewood, *Dalbergia sissoo*). Members of the Mimosoideae are characterized by their small, regular (actinomorphic) flowers which are generally crowded into spikes. *Acacia* species are the best known members of this family (Gutteridge and Shelton 1994). Many genera are economically important as sources of paper pulp (e.g. brown salwood, *Acacia mangium*), gum (e.g. gum Arabica, *Acacia senegal*), timber (e.g. Australian blackwood, *Acacia melanoxylon* and East Indian walnut, *Albizia lebbek*), tanning (e.g. golden wattle, *Acacia pycnantha*) and for bees (e.g. black locust, *Robinia pseudoacacia*).

Tropical tree legumes are also good sources of food, fodder, fuel-wood, resins and dyes. In addition, the plants are highly valuable in agroforestry

<sup>1</sup> Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore, e-mail: hongy@tll.org.sg

systems. Some important forage tree legumes include *Leucaena leucocephala*, *Gliricidium sepium*, *Calliandra calothyrsus*, *Sesbania sesban* and *Desmodium* species (Gutteridge and Shelton 1994). *Acacia mangium*, *Albizia lebbeck*, *Dalbergia sissoo*, *Prosopis cineraria* and *Robinia pseudoacacia* are fast growing and can withstand high temperatures, waterlogging and drought, and thus are most suitable for reforestation, reclamation of wastelands and as shelterbelts to check soil erosion. These trees may also play an important role in natural and cultivated ecosystems owing to their symbiotic relationship with bacteria of the family Rhizobiaceae, or with fungi through mycorrhizal associations to improve soil fertility. These multipurpose tropical tree legumes are thus the major targets of biotechnological research in agroforestry.

## 2 Progress and Prospects of Tree Legume Biotechnology

Conventional tree improvement is based on managing genetic resources, including selection for superior clones from existing forests, conservation of genetic variability, partially controlled propagation and classical breeding for desired traits. Traditional breeding methods involve sets of genes being introduced through sexual hybridization and this is restricted by sexual incompatibility manifested at the interspecific and intergeneric level, and related problems such as sterility and apomixes (Khurana and Khurana 1999). Because of the prevalence of high heterozygosity in forest trees, a number of recessive deleterious alleles are retained within the population, resulting in high genetic load and inbreeding depression. This limits the use of traditional breeding methods, such as selfing and back-crossing, and makes it difficult to fix desirable alleles in a particular genetic background (Williams and Savolainen 1996). Other limiting factors of conventional breeding methods include a long juvenile phase and life span, the requirement of space for large segregating populations and a lack of genetically pure lines.

Biotechnology holds considerable potential to overcome several limitations encountered in breeding and to accelerate forest tree breeding programs in several ways. These include rapid clonal propagation of superior genotypes by tissue culture techniques, production of somatic hybrids using protoplast fusion to overcome sexual incompatibility, generation of useful mutants using somaclonal variation, development of genetic markers to complement traditional breeding (marker assisted breeding) and the generation of novel plants with desirable traits by genetic transformation.

## 3 In Vitro Propagation of Legume Tree Species

Various tissue culture techniques, such as meristem culture, de novo shoot organogenesis, somaclonal variation, mutagenesis and somatic embryogene-

sis, have been exploited extensively for mass propagation and the generation of genetically modified forest and cultivated trees (Giri et al. 2004) including legumes (Khurana et al. 2003). Judicious selection and collection of explants, with appropriate use of plant growth regulators, antioxidants, additives and adsorbents, are prerequisites for developing reliable and efficient plant regeneration protocols. Tables 1–4 summarize the in vitro propagation work on legume tree species.

### 3.1 Meristem Culture

Axillary bud or shoot-tip culture is the most common method used for clonal propagation in plants, especially for woody species. It offers the advantages of rapid and easy development of multiple shoots, leading to the formation of true-to-parental-type plants in bulk. It has been used extensively for the propagation of *Acacia* species (Vengadesan et al. 2002), *Dalbergia sissoo* (Pradhan et al. 1998a), *Gleditsia triacanthos* (Vila et al. 1999), *Leucaena leucocephala* (Nangia and Singh 1996) and *Robinia pseudoacacia* (Davis and Keathley 1987) (Table 1). The success of a meristem propagation protocol depends on the appropriate combination of explants, culture medium and growth regulators.

#### 3.1.1 Age, Type and Source of Explants

Juvenile tissues are generally more responsive to in vitro manipulations compared to mature tissues. In trees, the juvenile characters may be preserved at the base (ontogenetically young tissue) (Giri et al. 2004). In the absence of such materials, some manipulations for reversal of age, such as partial rejuvenation, may be needed. In *Acacia mangium*, about 30% of the buds and/or shoot apices from the 3-m-tall trees and 18% from the 20-m-tall trees produced rejuvenated shoots after 4 months of culture on MS medium (Murashige and Skoog 1962) containing  $2 \text{ mg l}^{-1}$  benzyladenine (BA). There was no difference in rejuvenation between axillary buds and shoot apices (Xie and Hong 2002). *Acacia mangium* microshoots of juvenile origin rooted more freely than those from mature sources (Monteuuis and Bon 2000). Micropropagation in *Acacia catechu* has been reported using the shoot-tips of in vitro germinated 15-day-old seedlings (Kaur and Kant 2000) and from nodal explants from 10- to 20-year-old trees (Kaur et al. 1998). The response of explants and the requirement for growth regulators varied with genotype. Seasonal variation in explant collection may influence bud-break, the number of shoots per explant and microbial contamination. Amongst the various studies conducted in different species of *Acacia*, nodal segments were found to be the most suitable explants for multiple shoot formation (Vengadesan et al. 2002).

**Table 1.** Summary of meristem culture in tropical legume trees. WPM = woody plant medium (Lloyd and McCown 1980); BTM = broadleaf tree medium (Chalupa 1983)

Species	Explant	Medium/growth regulator (mg l <sup>-1</sup> )	Reference
<i>Acacia auriculiformis</i>	Axillary bud	MS + BA (1.1) + NAA (0.2)	Reddy et al. (1995)
<i>Acacia catechu</i>	Shoot-tip	MS + BA (1.5) + KT (1.5)	Kaur and Kant (2000)
	Nodal segment	MS + BA (4) + NAA (0.5)	Kaur et al. (1998)
<i>Acacia mangium</i>	Axillary bud and shoot-tip	MS + BA (2)	Xie and Hong (2002)
	Nodal segment	B5 + BA (1) + IBA (0.5)	Bon et al. (1998)
<i>Acacia mearnsii</i>	Axillary bud	MS + BA (2) + IAA (0.01)	Huang et al. (1994)
<i>Acacia nilotica</i>	Cotyledonary node	B5 + BA (1.5)	Dewan et al. (1992)
<i>Ceratonia siliqua</i>	Nodal segment	MS + zeatin (1.1)	Sebastian and McComb (1986)
<i>Cercis canadensis</i>	Shoot-tip	WPM + BA (5)	Bennett (1987)
<i>Cercis siliquastrum</i>	Shoot-tip, apical and lateral buds	WPM + BA (2.3)	Grubisic and Culafic (1986)
<i>Dalbergia sissoo</i>	Nodal segment	MS + BA (1) + NAA (0.1)	Gulati and Jaiwal (1996)
	Cotyledonary node	MS + BA (2)	Pradhan et al. (1998a)
<i>Gleditsia triacanthos</i>	Axillary bud and nodal segment	MS + KT (0.05) + IAA (3) <sup>a</sup>	Vila et al. (1999)
<i>Leucaena leucocephala</i>	Cotyledonary node and nodal segment	MS + BA (0.5–2)	Nangia and Singh (1996)
<i>Mimosa tenuiflora</i>	Axillary bud	MS + KT (3) + IAA (0.1)	Villarreal and Rojas (1996)
<i>Paraserianthes falcataria</i>	Nodal segment	MS + BA (0.5) + NAA (0.3)	Bon et al. (1998)
<i>Prosopis alba</i>	Nodal segment	MS + KT (0.05) + NAA (3)	De-Meier and Bovo (2000)
<i>Prosopis chilensis</i>	Nodal segment	BTM + BA (0.05) + IAA (3)	Caro et al. (2002)
<i>Pterocarpus marsupium</i>	Cotyledonary node	MS + BA (1) + NAA (0.05)	Chand and Singh (2004)
<i>Pterocarpus santalinus</i>	Nodal segment	B5 + BA (2)	Anuradha and Pullaiah (1999)
<i>Robinia pseudoacacia</i>	Axillary bud	MS + BA (0.07)	Davis and Keathy (1987)
<i>Sophora toromiro</i>	Embryonic shoot-tip	WPM + BA (0.1) + NAA (0.3)	Jordan et al. (2001)

<sup>a</sup> Plants not formed



### 3.1.2 Culture Medium

Various media such as MS, B5 (Gamborg et al. 1968), SH (Schenk and Hilderbrant 1972), WPM (woody plant medium; Lloyd and McCown 1980) and BTM (broadleaf tree medium; Chalupa 1983) have been used in legume tree culture. These media have resulted in significant differences in culture responses, including the number of explants producing shoots, the growth of axillary shoots, the number of shoots producing roots and the number and length of roots. MS medium was optimal in most of the tree legumes such as *Acacia salicina*, *Acacia saligna* and *Acacia sclerosperma* (Jones et al. 1990), *Acacia mangium* (Xie and Hong 2002), *Acacia catechu* (Kaur and Kant 2000), *Acacia mearnsii* (Huang et al. 1994), *Acacia sinuata* (Vengadesan et al. 2002), *Dalbergia sissoo* (Pradhan et al. 1998a), *Leucaena leucocephala* (Nangia and Singh 1996) and *Mimosa tenuiflora* (Villarreal and Rojas 1996), while diluted MS medium (50 or 75% strength) gave an improved culture response with respect to clonal propagation of *Acacia mangium*, *Acacia senegal*, *Acacia albida* (Vengadesan et al. 2002) and *Paraserianthes falcataria* (formerly known as *Albizzia falcataria*) (Bon et al. 1998). B5 medium was shown to be optimal in *Acacia auriculiformis* (Mittal et al. 1989) and *Acacia nilotica* (Dewan et al. 1992), while 75% strength B5 resulted in taller shoots for *Acacia mangium* (Bon et al. 1998). WPM and BTM were used for the culture of *Cercis canadensis* (Bennett 1987) and *Prosopis chilensis* (Caro et al. 2002), respectively.

### 3.1.3 Effect of Growth Regulators

The manipulation of plant cells, tissues and organs in culture for micropropagation and genetic modifications is highly dependent on the use of appropriate growth regulators. Various combinations of these hormones have led to successful shoot formation in different tree legumes. Amongst the growth regulators, BA ( $0.5\text{--}5\text{ mg l}^{-1}$ ) is the most commonly used cytokinin, with or without auxin or the additional cytokinins kinetin (KT)/zeatin (Z) in the medium. It has been reported that the combination of BA ( $2\text{ mg l}^{-1}$ ) and indole-3-acetic acid (IAA,  $0.01\text{ mg l}^{-1}$ ) was most effective for inducing shoot proliferation in *Acacia mearnsii* (Huang et al. 1994), with BA ( $1\text{ mg l}^{-1}$ ) and indole-3-butyric acid (IBA,  $0.5\text{ mg l}^{-1}$ ) for *Acacia mangium* (Bon et al. 1998). In *Dalbergia sissoo*, BA ( $2\text{ mg l}^{-1}$ ) induced high-frequency shoot proliferation (99%) and the maximum number of shoots per explant (7.9 shoots); 60–70 shoots were obtained within 3 months from a single cotyledonary node (Pradhan et al. 1998a). Thidiazuron (TDZ) alone was shown to be more efficient than cytokinins and auxins in shoot formation, but the concentration and duration of TDZ treatment was critical. Explants grown on medium with TDZ ( $0.1\text{ mg l}^{-1}$ ) for 1 month showed increased shoot proliferation in 60% of the nodal segments of *Acacia mangium*, but shoots needed to be transferred to TDZ-free medium containing BA ( $2\text{ mg l}^{-1}$ ) + GA<sub>3</sub> ( $0.5\text{ mg l}^{-1}$ ) for shoot growth (Bhatnagar and

Hong, unpublished data). TDZ was also found to stimulate shoot proliferation in *Acacia sinuata*, but prolonged culture in TDZ-containing medium resulted in stunted growth which was overcome after transfer to medium with GA<sub>3</sub> (0.6 mg l<sup>-1</sup>) (Vengadesan et al. 2002). In *Sophora toromiro*, embryogenic shoot-tips produced a single shoot per explant on WPM containing BA (0.1 mg l<sup>-1</sup>) and NAA (0.3 mg l<sup>-1</sup>); however, in whole embryonic axis explants the presence of TDZ (0.5–2.5 mg l<sup>-1</sup>) during subculture induced multiple shoot formation and profuse branching of the elongating shoots. Similarly, the callus formed at the base of the embryogenic axis developed into several new shoots after 60–70 days of culture in the presence of TDZ (Jordan et al. 2001).

A common phenomenon in cultures of mature explants is the release of tannins and phenolics in the medium, resulting in browning of the medium and a decrease in the shoot regeneration frequency. To overcome tissue browning, several compounds such as activated charcoal as adsorbent and polyvinylpyrrolidone (PVP), ascorbic acid and citric acid as antioxidants were added to the culture medium. The addition of activated charcoal at 2 g l<sup>-1</sup> improved shoot multiplication, reduced leaf chlorosis, and increased the percentage of elongated shoots and percentage rooting in nodal segments of *Acacia mearnsii* (Quoirin et al. 2001) and at 2.5 g l<sup>-1</sup> in shoot-tip cultures of *Cercis canadensis* (Mackay et al. 1995). Ascorbic acid (20 mg l<sup>-1</sup>) proved beneficial in *Acacia catechu* (Kaur et al. 1998), while citric acid and PVP were less effective in *Acacia sinuata* (Vengadesan et al. 2002).

### 3.2 Organogenesis

Differentiation via de novo organization of meristems is referred to as organogenesis. This process can be either direct or indirect via an intermediate callus phase. The formation of shoots directly from the tissue sections lacking a pre-formed meristem (i.e. adventitious origin) is the preferred mode of clonal propagation, as it does not involve somaclonal variation. However, a regenerative callus could be a constant source of new plants and, at the same time, can serve to maintain regenerative cell lines.

As summarized in Table 2, direct organogenesis has been reported in *Dalbergia sissoo* where shoot bud differentiation occurred after 15–20 days of culture on MS medium containing BA (1 mg l<sup>-1</sup>) and  $\alpha$ -naphthaleneacetic acid (NAA, 0.05 mg l<sup>-1</sup>) from semi-mature cotyledons (52%) and after 25–30 days in the presence of BA (5 mg l<sup>-1</sup>) in mature cotyledons (8%). Pretreatment of mature cotyledons with BA (2 mg l<sup>-1</sup>) for 48 h markedly enhanced adventitious shoot formation (45%) (Singh et al. 2002). Shoot buds differentiated directly on MS medium with BA (1 mg l<sup>-1</sup>) from hypocotyls of *Tamarindus indica* (Sonia et al. 1998) and cotyledons of *Sesbania grandifolia* (Detrez et al. 1994). Khattar and Mohan Ram (1982) reported the direct regeneration of shoot buds in hypocotyl explants of *Sesbania sesban* cultured on B5 medium

containing BA ( $1 \text{ mg l}^{-1}$ ), and indirect organogenesis on B5 medium with BA ( $1.2\text{--}2.2 \text{ mg l}^{-1}$ ), NAA ( $0.2\text{--}1 \text{ mg l}^{-1}$ ) and GA<sub>3</sub> ( $0.35\text{--}1.75 \text{ mg l}^{-1}$ ).

The occurrence of indirect organogenesis is more common (Table 2) and different combinations of auxins and cytokinins are often used for induction of callus formation, adventitious bud formation, shoot elongation and rooting. TDZ that mimics both auxin and cytokinin responses has been used to promote micropropagation, induce shoot organogenesis and somatic embryogenesis, and help in the recovery of transgenic plants. Its effectiveness and stability, coupled with its requirement at low concentration and for a short duration, makes TDZ the most potent growth regulator for woody plant cultures. Application of TDZ has rendered many recalcitrant woody plants more amenable to biotechnological manipulations (Khurana et al. 2005).

### 3.2.1 Callus Formation

In tree legumes, callus formation can be induced from various explants, such as hypocotyls, cotyledons, roots, internodes, leaves and floral organs. It has been reported that a combination of KT ( $1 \text{ mg l}^{-1}$ ) with NAA ( $1 \text{ mg l}^{-1}$ ) and 2,4-dichlorophenoxyacetic acid (2,4-D) at  $0.5$  or  $1 \text{ mg l}^{-1}$  promoted callus formation from explants of *Albizia lebbek* (Rao and De 1987). The presence of BA ( $1 \text{ mg l}^{-1}$ ) in combination with IAA ( $0.1 \text{ mg l}^{-1}$ ) resulted in callus formation in 100% of stem and 36% of petiole explants of *Albizia lebbek*. In *Cassia fistula* and *Cassia siamea*, 50% of stem explants also formed calli on the same medium, but petioles responded poorly (Gharyal and Maheshwari 1990). It was found that 2,4-D ( $2 \text{ mg l}^{-1}$ ) and KT ( $3 \text{ mg l}^{-1}$ ) promoted callus formation from 100% of cotyledons (from zygotic embryos), leaves, petioles and stems from 50-day-old seedlings of *Acacia mangium* (Xie and Hong 2001a).

### 3.2.2 Induction of Adventitious Buds

Shoot bud formation was observed from 50% of *Albizia julibrissin* root explants grown on hormone-free B5 medium within 15 days of culture. The addition of TDZ ( $0.01 \text{ mg l}^{-1}$ ) markedly increased the regeneration frequency up to 95%, with each explant forming about 10 shoots (Sankhla et al. 1996). In *Albizia lebbek* the presence of BA at  $1 \text{ mg l}^{-1}$  induced high frequency shoot regeneration (96%) from hypocotyl-derived callus, with each explant forming 25 shoot buds (Rao and De 1987). Gharyal and Maheshwari (1990) reported that a combination of BA ( $1 \text{ mg l}^{-1}$ ) and IAA ( $0.5 \text{ mg l}^{-1}$ ) promoted shoot formation from stem (50%)- and petiole (36%)-derived calli, while BA ( $4 \text{ mg l}^{-1}$ ) and 2,4-D ( $2 \text{ mg l}^{-1}$ ) resulted in multiple shoot formation in root-derived calli of *Albizia lebbek* (Miah and Rao 1996). In *Acacia auriculiformis*, the combination of BA ( $1 \text{ mg l}^{-1}$ ) with NAA ( $0.5 \text{ mg l}^{-1}$ ) promoted adventitious bud formation from callus (Rao and Prasad 1991). On B5 medium containing BA ( $1 \text{ mg l}^{-1}$ ) and IAA ( $0.5 \text{ mg l}^{-1}$ ), stem and peti-

Table 2. Regeneration through organogenesis (callus formation and adventitious shoot induction) in tropical legume trees

Species	Explant	Media/growth regulator (mg l <sup>-1</sup> ) for callus induction	Media/growth regulator (mg l <sup>-1</sup> ) for shoot bud induction	Reference
<i>Acacia mangium</i>	Cotyledon (from zygotic embryo), leaf, petiole and stem	MS + 2,4-D (2) + KT (3)	MS + TDZ (1) + IAA (0.25)	Xie and Hong (2001a)
<i>Albizzia lebeck</i>	Hypocotyl	MS + NAA (1) + KT (1)	MS + BA (1)	Rao and De (1987)
	Leaf	MS + 2,4-D (0.5) + KT (1)	MS + BA (5)	
	Stem	MS + 2,4-D (1) + KT (1)	MS + BA (5)	
	Stem and petiole	B5 + BA (1) + IAA (0.5)	B5 + BA (1) + IAA (0.5)	
<i>Albizzia procera</i>	Epicotyl and hypocotyl	1/2 MS + BA (0.003)	1/2 MS + BA (0.003)	Gharyal and Maheshwari (1990)
<i>Albizzia richardiana</i>	Hypocotyl	B5 + BA (2.2)	B5 + BA (2.2)	Swamy et al. (2004)
<i>Cassia fistula</i> ,	Stem and petiole	B5 + BA (1) + IAA (0.5)	B5 + BA (1) + IAA (0.5)	Tomar and Gupta (1988)
<i>Cassia siamea</i>				Gharyal and Maheshwari (1990)
<i>Cassia obtusifolia</i>	Cotyledon	MS + NAA (0.15)	MS + NAA (0.15) + BA (0.5) + KT (0.5) + zeatin (0.5)	Zhou et al. (2001)
<i>Dalbergia latifolia</i>	Leaf	MS + BA (1) + NAA (5)	MS + BA (5) + NAA (0.5)	LaxmiSita and Raghavaswamy (1993)
	Hypocotyl	MS + BA (0.5) + NAA (2)	MS + BA (3)	Pradhan et al. (1998b)
<i>Dalbergia sissoo</i>	Semi-mature cotyledon	No callusing	BA (1) + NAA (0.05) <sup>a</sup>	Singh et al. (2002)
	Cotyledon	No callusing	BA (5) <sup>a</sup>	
	Hypocotyl-derived cell suspension	MS + BA (2) + NAA (0.5)	MS + BA (3) + NAA (0.5)	
<i>Prosopis tamarugo</i>	Cotyledon and hypocotyl	MS + NAA (2)	BA (1-2.5)	Nandwani and Ramawat (1992)
<i>Robinia pseudoacacia</i>	Cambial tissue	MS + BA (1.0) + NAA (2)	MS + BA (5)	Han et al. (1993a)

Table 2. (continued)

Species	Explant	Media/growth regulator (mg l <sup>-1</sup> ) for callus induction	Media/growth regulator (mg l <sup>-1</sup> ) for shoot bud induction	Reference
<i>Sesbania sesban</i>	Hypocotyl	B5 + BA (1.2-2.2) + NAA (0.2-1)	B5 + BA (1.2-2.2) + NAA (0.2-1)	Khattar and Mohan Ram (1982)
	Hypocotyl	No callusing	+ GA <sub>3</sub> (0.35-1.75)	
<i>Sesbania grandiflora</i>	Cotyledon	No callusing	B5 + BA (1.2-2.2) <sup>a</sup>	Khattar and Mohan Ram (1982)
<i>Sesbania</i> spp.	Hypocotyl	No callusing	MS + BA (1) <sup>a</sup>	Detrez et al. (1994)
		MS + BA (1-2) + IBA (0.05-0.1)	MS + BA (1-2) + IBA (0.05-1)	Yan-Xiu et al. (1993)
<i>Tamarindus indica</i>	Hypocotyl	No callusing	MS + BA (1) <sup>a</sup>	Sonia et al. (1998)
	Embryo axis	MS + BA (10) + NAA (0.5)	MS + BA (0.5) + zeatin (0.2)	Mehta et al. (2000)

<sup>a</sup>Direct organogenesis

olar calli of *Cassia fistula* and *Cassia siamea* turned brown after a month, but the addition of PVP facilitated recovery of shoots from stem explants (Gharyal and Maheshwari 1990). A combination of TDZ ( $1 \text{ mg l}^{-1}$ ) with IAA ( $0.25 \text{ mg l}^{-1}$ ) promoted the formation of adventitious shoots in 8–11% of calli initiated from cotyledon, leaf or petiole explants and a small number of stem-derived calli (4%) of *Acacia mangium*, after 40 days of culture (Xie and Hong 2001a).

### 3.2.3 Shoot Elongation

After adventitious buds are induced, shoot buds are usually transferred to the medium with lower concentrations of cytokinins, with  $\text{GA}_3$  and/or auxin for elongation. In *Acacia mangium*,

the medium with a low TDZ concentration ( $0.01 \text{ mg l}^{-1}$ ) and  $\text{GA}_3$  ( $2.5 \text{ mg l}^{-1}$ ) promoted shoot elongation (Xie and Hong 2001a). According to Mehta et al. (2000), zeatin ( $0.2 \text{ mg l}^{-1}$ ) and BA ( $0.5 \text{ mg l}^{-1}$ ) are recommended for shoot elongation of *Tamarindus indica*.

### 3.2.4 Rooting of Regenerated Shoots

The presence of cytokinins in the shoot induction medium has been shown to inhibit root formation from regenerated shoots. In general, media free of hormone or those supplemented with auxins are used for rooting. In *Tamarindus indica*, shoots cultured for 72 h in the presence of IBA, indole-3-pyruvic acid (IPA) and NAA, each at  $0.4 \text{ mg l}^{-1}$ , prior to transfer to a hormone-free medium, promoted rooting (Mehta et al. 2000). B5 medium with or without IAA ( $0.1 \text{ mg l}^{-1}$ ) was found to be suitable for rooting of *Albizia lebbeck*, *Cassia fistula* and *Cassia siamea* (Gharyal and Maheshwari 1990). In *Albizia lebbeck*, the poor rooting response on MS medium was improved by transferring to Bonner's inorganic nutrient medium (lacking a carbon source) supplemented with IBA ( $2 \text{ mg l}^{-1}$ ) (Rao and De 1987). Up to 80% of shoots regenerated from immature cotyledons and 56% from mature cotyledons of *Dalbergia sissoo* formed roots in the presence of 0.25 and  $1 \text{ mg l}^{-1}$  IBA, respectively (Singh et al. 2002). Results of our study showed that NAA ( $2 \text{ mg l}^{-1}$ ) and KT ( $0.5 \text{ mg l}^{-1}$ ) promoted rooting in 10% of the elongated shoots of *Acacia mangium* (Xie and Hong 2001a). The rooting frequency was increased to 25% in the presence of IAA ( $1 \text{ mg l}^{-1}$ ) and activated charcoal (0.1–1.0%) (Bhatnagar and Hong, unpublished data). The promoting effect of activated charcoal on rooting may be attributed to its adsorbing the high concentrations of endogenous cytokinin in cultured shoots and blocking the light from reaching the roots through the medium (George 1993).

### 3.3 Somatic Embryogenesis

Somatic embryogenesis offers the capability to produce unlimited number of propagules and artificial seeds. Compared to plant regeneration through organogenesis, somatic embryogenesis has the advantage of single cell regeneration, high multiplication rate and the formation of bipolar structures with easy rooting. Currently, it appears to be the most promising approach to introduce genes of interest into herbaceous legumes. However, the disadvantage of this approach is the possible phenotypic segregation of plants derived from different seeds, which is an important issue for tree legumes.

As summarized in Table 3, hormone-free B5 medium induced direct somatic embryo development from hypocotyls of *Albizzia lebbeck* (Gharyal and Maheshwari 1981). Short-duration exposure to red and far-red light enhanced considerably the number of explants producing somatic embryos (90%) in *Albizzia lebbeck*, with each hypocotyl explant forming 12–14 somatic embryos. This suggested a possible involvement of phytochrome in somatic embryo development (Baweja et al. 1995). On B5 medium containing BA ( $2.2 \text{ mg l}^{-1}$ ), the hypocotyl explants of *Albizzia richardiana* showed callus differentiation via somatic embryogenesis at 2% sucrose levels and shoot formation via organogenesis on sucrose 4% (Tomar and Gupta 1988). In *Calliandra tweedii*, up to 100 embryos per explant could be differentiated directly from internodal segments in the presence of isopentenyl adenine (2iP,  $0.2 \text{ mg l}^{-1}$ ) and from petioles at  $1 \text{ mg l}^{-1}$  NAA, but 2,4-D at  $0.02\text{--}2 \text{ mg l}^{-1}$  induced somatic embryos via a callus stage in both explants (Kumar et al. 2002).

Shahana and Gupta (2002) compared different basal media and found that LS medium (Linsmaier and Skoog 1965) fortified with NAA ( $0.1 \text{ mg l}^{-1}$ ) was best for induction of somatic embryos from cotyledon-derived calli of *Sesbania sesban*. We found that a combination of TDZ ( $2 \text{ mg l}^{-1}$ ) and IAA ( $0.25 \text{ mg l}^{-1}$ ) induced somatic embryos from immature cotyledon-derived calli of *Acacia mangium* (Xie and Hong 2001b). However, with respect to somatic embryo induction, the medium with KT ( $3 \text{ mg l}^{-1}$ ) in combination with NAA ( $0.5 \text{ mg l}^{-1}$ ) was optimal for *Acacia catechu* (Rout et al. 1995) and with BA ( $5.5 \text{ mg l}^{-1}$ ) and 2,4-D ( $2.2 \text{ mg l}^{-1}$ ) for immature endosperm culture of *Acacia nilotica*, and resulted in triploid plants (Garg et al. 1996). As significant morphological variations among *Acacia* species have been documented (Simmons 1987), the differential requirement for plant growth regulators according to different species is not surprising. Somatic embryogenesis has also been reported in tree legumes such as *Acacia koa* (Skolmen 1986), *Cercis canadensis* (Trigiano et al. 1988; Geneve and Kester 1990), *Robinia pseudoacacia* (Merkle and Wiecko 1989), *Genista monosperma* (Curir et al. 1990) and *Cladrastis lutea* (Weaver and Trigiano 1991).



Table 3. Summary of somatic embryogenesis in tropical legume trees

Species	Explant	Somatic embryo induction medium (mg l <sup>-1</sup> )	Shoot formation medium	Reference
<i>Acacia catechu</i>	Immature cotyledon	WPM + KT (3) + NAA (0.5)	1/2 MS	Rout et al. (1995)
<i>Acacia mangium</i>	Immature cotyledon	MS + TDZ (2) + IAA (0.25)	1/2 MS + GA <sub>3</sub> (5)	Xie and Hong (2001b)
<i>Acacia nilotica</i>	Immature endosperm	MS + BA (5.5) + 2,4-D (2.2)	MS	Garg et al. (1996)
<i>Albizzia julibrissin</i>	Ovule	MS + 2,4-D (10)	MS	Burns and Wetzstein (1998)
<i>Albizzia lebbeck</i>	Hypocotyl and immature cotyledon	B5	B5 <sup>a</sup>	Gharyal and Maheshwari (1981)
<i>Calliandra tweedii</i>	Internode	MS + 2ip (0.2)	MS + 2ip (0.2) <sup>a</sup>	Kumar et al. (2002)
	Petiole	MS + NAA (1)	MS + NAA (1) <sup>a</sup>	
<i>Ceratonia siliqua</i>	Fertilized ovule	MS + 2,4-D (1)	1/2MS + IBA (2)	Carimi et al. (1997)
<i>Cercis canadensis</i>	Immature zygotic embryo	SH + 2,4-D (3)	SH + 2,4-D (3)	Trigiano et al. (1988)
	Immature zygotic embryo	WPM + 2,4-D (1.1)	WPM + 2,4-D (1.1)	Geneve and Kester (1990)
<i>Dalbergia sissoo</i>	Zygotic embryo	MS + KN (0.1–0.25) + 2,4-D (1.5–2)	1/2 MS	Das et al. (1997)
<i>Sesbania sesban</i>	Cotyledon	LS + NAA (0.1)	LS	Shahana and Gupta (2002)

<sup>a</sup> Direct somatic embryogenesis

### 3.4 Protoplast Culture

Protoplasts can be used for the production of somatic hybrids through fusion. Protoplasts are also source material for genetic transformation. Saxena and Gill (1987) reported the isolation of 90% viable protoplasts of *Pithecellobium dulce* using an enzyme mixture consisting of 1% (w/v) cellulase, 0.25% pectolyase, 0.5% macerozyme, 0.5% rhozyme, 0.3 M sorbitol and 0.3 M glucose. Pretreatment of tissues by overnight incubation of excised leaves at 10 °C in liquid shoot culture medium in the absence of sucrose was required to obtain viable protoplasts. Up to 3% of the cells grown in medium with 2,4-D (0.5 mg l<sup>-1</sup>), NAA (0.5 mg l<sup>-1</sup>) and BA (0.5 mg l<sup>-1</sup>) formed calli. After the calli were transferred to medium with BA (1.25 mg l<sup>-1</sup>) and IAA (0.17 mg l<sup>-1</sup>), 10–15% gave rise to shoots, which were rooted in the presence of 0.17 mg l<sup>-1</sup> IAA (Saxena and Gill 1987). In *Acacia mangium*, protoplasts could not be isolated using conventional cell wall degrading enzymes such as cellulase and pectolyase, but, instead,  $\beta$ -1,3-glucanases yielded viable protoplasts (Toshihiro and Sonoko 1999). Protoplast isolation and culture were attempted in *Robinia pseudoacacia* (Han and Keathley 1988) and *Leucaena leucocephala* (Venketeshwaran and Gandhi 1982), but plant regeneration was not reported.

## 4 Genetic Transformation

Genetic transformation complements plant breeding efforts by increasing the diversity of genes and germplasm available for incorporation into desirable plant species. The methodology allows the introduction of genes of interest into the plant in a single step for precision breeding, thereby shortening the time period required for the production of new varieties. Recent advances in legume transformation have been reviewed by Somers et al. (2003). However, compared with the rapid progress in herbaceous legume species, there are few reports of genetic transformation of tree legumes. The lack of reliable and reproducible regeneration protocols, coupled with the complicated biology of *Agrobacterium*/host plant interaction and the general tolerance of plant tissues to kanamycin, are the main contributing factors retarding the utilization of transformation techniques in tropical legume tree species (Table 4). It is imperative to develop an efficient transformation system independent of genotype for the routine production of transgenic plants. The system should not depend on extensive manipulation of tissue culture techniques, such as protoplast culture and embryogenic suspension cultures, to prevent culture-induced mutations and somaclonal variation.

Table 4. Summary of genetic transformation in tropical legume trees

Species	Transformation method	Explant	Growth regulator for shoot induction/selection agent (mg l <sup>-1</sup> )	Result	Reference
<i>Albizia lebbek</i>	Particle bombardment	Hypocotyl	Not cultured	Transient expression, no transgenic plants	Khurana and Khurana (2000)
<i>Acacia mangium</i> , <i>Acacia mearnsii</i>	Particle bombardment	Hypocotyl and cotyledon	Not cultured	Transient expression, no transgenic plants	Quoirin et al. (2002)
<i>Acacia mangium</i>	<i>Agrobacterium tumefaciens</i>	Stem segment	TDZ (1) + IAA (0.25) + geneticin (12–15)	Transgenic plants	Xie and Hong (2002)
<i>Robinia pseudoacacia</i>	<i>Agrobacterium rhizogenes</i>	Leaf disc	BA (1.25) + NAA (1.75) + kanamycin (100)	Transgenic plants with abnormalities	Han et al. (1993b)
<i>Robinia pseudoacacia</i>	<i>Agrobacterium tumefaciens</i>	Leaf disc and stem segment	BA (2.5) + zeatin (2.5) + 2,4-D (0.05) + hygromycin (20)	Transgenic plants	Igasaki et al. (2000)

#### 4.1 Gene Delivery via Particle Bombardment

The ability to deliver DNA-coated particles (microprojectiles) directly into intact tissue by particle bombardment has expanded the range of organisms that can be genetically transformed. In *Albizzia lebbek*, bombardment of hypocotyl explants with gold particles coated with pBI1221, which carried the *gus* gene encoding  $\beta$ -glucuronidase under the control of the cauliflower mosaic virus 35S promoter, resulted in transient *gus* expression in bombarded tissues, with up to 35–200 GUS positive spots per explant (Khurana and Khurana 2000). It has been reported that in tissues bombarded with tungsten particles coated with a construct with the *gus* gene under the control of an enhanced 35S (e35S) promoter, GUS expression was about nine times higher than when the *gus* gene was driven by 35S promoter in the hypocotyl of *Acacia mearnsii*, and five times higher in cotyledons of *Acacia mangium* (Quoirin et al. 2000). High acceleration pressure (1,550 psi) and osmotic treatment with mannitol and sorbitol (0.3 or 0.4 M each) for 3–4 h before bombardment significantly increased transgene expression in *Acacia mearnsii* (Quoirin et al. 2002). However, transgenic plants were not reported in these studies.

#### 4.2 *Agrobacterium*-Mediated Transformation

Among the various transformation methods, *Agrobacterium*-mediated transfer of foreign DNA has been most commonly used. This method offers several advantages, including defined integration of transgenes, relatively low copy numbers, and preferential integration into transcriptionally active regions of the chromosomes. The ability to transform tissues and to regenerate transgenic plants depends on several factors. These include the virulence of *Agrobacterium* and density of the bacterial suspension, susceptibility of the host plant to *Agrobacterium*, the period of co-cultivation, plant phenolics secreted in response to wounding, the efficiency of selection that allows the growth of transformed cells, and the frequency of shoot regeneration from transformed cells (Bhatnagar et al. 2004).

Transgenic *Acacia mangium* plants were obtained via cocultivation of stem segments (from in vitro rejuvenated shoots) with *Agrobacterium tumefaciens* strain LBA4404 carrying pBI121, followed by a five-step selection on the medium containing TDZ ( $1.0 \text{ mg l}^{-1}$ ), IAA ( $0.25 \text{ mg l}^{-1}$ ), geneticin ( $12\text{--}30 \text{ mg l}^{-1}$ ) and timentin ( $50\text{--}300 \text{ mg l}^{-1}$ ) (Xie and Hong 2002). Genomic Southern blot hybridization confirmed the incorporation of the *nptII* gene encoding neomycin phosphotransferase II into the host genome. As a selection agent, kanamycin, even at a high concentration of  $300 \text{ mg l}^{-1}$ , was ineffective in *Acacia mangium* (Xie and Hong 2002). In *Robinia pseudoacacia*, hygromycin B at low concentrations was found to be more effective in killing leaf discs than kanamycin at similar concentrations, while geneticin had an intermediate effect (Igasaki et al. 2000).

Transgenic plants of *Robinia pseudoacacia* were also obtained via *Agrobacterium rhizogenes*-mediated transformation. However, plants exhibited growth abnormalities, such as wrinkled and variegated leaves, reduced apical dominance, reduced internode length, asymmetric leaflets and reduced spine length, after 3 months of growth (Han et al. 1993b). These abnormalities may be due to high concentrations of cytokinin in the tissues. This problem may be overcome by the use of a partially disarmed *Agrobacterium rhizogenes* strain (in which *rol* genes are deleted). In contrast, transgenic *R. pseudoacacia* plants with a normal phenotype were produced via *Agrobacterium tumefaciens*-mediated transformation (Igasaki et al. 2000). This is a significant achievement, as genetic engineering should allow selective improvement of a single trait without the loss of the desired traits of the parental line.

### 4.3 Traits for Legume Tree Engineering

The trunk is the major harvested organ of legume tree species and hence the main target for improvement of plant performance and wood quality. The latter includes the biochemical modification of wood features such as the content or composition of lignins, cellulose quality and content, branching architecture and growth rate. Other target traits include improved root systems, increased nitrogen fixation capability, the production of useful secondary metabolites, herbicide and insect resistance, and abiotic stress tolerance to drought and flooding. It may be important to introduce a sterility mechanism into genetically modified trees to address the issue of gene flow, especially for perennial legume tree species, as pollen and seeds may travel long distances.

### 4.4 Obstacles to the Genetic Engineering of Tree Legumes

Although it is possible to genetically transform tree legumes, some problems remain to be addressed. These include obstacles for the commercialization of genetically modified legume trees. Transformation protocols are available for only a few species and they generally suffer from low efficiency, chimerism of transgenic plants and insufficient number of transgenic plants for field evaluation. Transformation efficiency may be improved by several strategies as follows:

1. Increased *Agrobacterium*-mediated DNA delivery by reducing or overcoming factors that inhibit host–pathogen interaction. This may be achieved by the development of superbinary vectors and hypervirulent strains. Recently, adding thiol compounds in the cocultivation medium was found to increase the transformation efficiency in soybean (Olhoft et al. 2001). This increase seems to be mediated via thiol inhibition of peroxidase and polyphenol oxidase in the explants. It would be interesting to determine whether thiol compounds also improve the transformation of tree legumes.

2. Optimized selection and identification systems. The use of various selection markers other than kanamycin should be investigated. Selection for hygromycin tolerance decreased the percentage of non-transformed escapes in soybean (Olhoft et al. 2003).
3. Use the meristems as targets for transformation. This approach was successful in the production of transgenic soybean plants via *Agrobacterium*-mediated transformation (Hinchey et al. 1988). It is postulated that the amiability of meristems to transformation is less limited by genotype. Use of meristem cultures for genetic transformation in tree legumes has the potential to expand the range of species/genotypes that can be transformed and to shorten the time for transgenic plant production.
4. With the various difficulties associated with tissue culture of legume trees, it is tempting to pursue non-tissue culture transformation systems, such as the floral dip method as used in *Arabidopsis*. The success of a seedling and flower *Agrobacterium* infiltration method for *Medicago truncatula* paved the way for high throughput genomic studies in this legume (Trieu et al. 2000). Besides the technical difficulties in transgenic legume tree production, there is uncertainty of the stability of a promoter and construct in the extended life cycle of trees. Unique constructs with more suitable promoters, preferably of tree origin, are probably required for long-term expression of foreign genes in the trees.

## 5 Marker Assisted Breeding

Marker assisted breeding uses genetic fingerprinting techniques to assist breeders in matching genetic markers with the physical properties of a variety. The use of genetic markers can significantly accelerate the speed of natural plant breeding programs without exposure to the unpredictable health and environmental risks associated with genetic engineering techniques. DNA markers are polymorphic and the methods used to define DNA markers include randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLPs), microsatellites or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). Linkage analysis, association analysis and analysis of gene function can be used to determine the association of markers with desirable traits.

Using RAPD to analyze the effects of salt stress on plant growth and osmotic potential of leaves in *Acacia auriculiformis* and *Acacia mangium*, Nguyen et al. (2004) demonstrated that salt tolerance could be influenced by the genetic background of the provenances. However, further studies are needed to determine the association between RAPD markers and salt tolerance in these *Acacia* species.

Genetic linkage maps provide an important tool for the localization of genes linked to traits of economic importance, which could be used in marker assisted

selection. An integrated genetic linkage group map, comprised of 219 RFLP and 33 microsatellite loci in 13 linkage groups, was constructed by Butcher and Moran (2000) using two pedigrees of *Acacia mangium*. The heterogeneity in recombination frequencies did not result in major differences in the ordering of loci between pedigrees. Hence, the integrated map provides a sound basis for marker assisted selection in *Acacia mangium*. It also provides a reference map for comparative genome analysis in acacias. The co-dominant markers used for mapping are useful resources in population studies and for quality control in *Acacia* breeding programs.

## 6 Genomic and Molecular Biology Studies

Wide utilization of legumes, together with the variety of their symbiotic and pathogenic interactions with microorganisms, provides numerous targets for molecular and genomic studies. There were 989,679 nucleotide sequences from Fabaceae deposited in the National Center for Biotechnology Information in 2004 ([www.ncbi.nlm.nih.gov/taxonomy](http://www.ncbi.nlm.nih.gov/taxonomy)). Most of the studies were based on *Medicago truncatula*, *Lotus japonicus* and *Glycine max*, which accounts for 92.5% of the total entries in the Fabaceae. In comparison, information on the molecular biology of legume tree species is limited, with only 1160 sequences available in the Mimosoideae.

Little is known about genes involved in various important developmental processes, such as flower and wood development. Knowledge of these processes is particularly important for the exploitation of genetic modifications in trees. Transformation technology has raised the issue of environmental safety and possible gene flow. There is also a concern that transgenes from genetically modified trees may be passed on to natural forests, as trees live much longer than herbaceous species, and birds and insects can carry pollen and seeds over long distances. Effective gene containment is therefore necessary before genetically modified trees can be used in commercial plantations. This may be achieved by the generation of reproductive sterile plants through genetic ablation in floral organs, as demonstrated in *Populus*, *Nicotiana* and *Arabidopsis*, where expression of the cytotoxin gene was under the control of the promoter of the *Populus* floral specific *PTD* gene, which is a homologue of the MADS box genes *DEFICIENS* and *APETALA* (Skinner et al. 2003).

Our knowledge of flower development at the molecular level is derived mainly from studies of the herbaceous model plants *Arabidopsis thaliana* and *Antirrhinum majus*. Trees differ from these plant species as they are adapted to survive for much longer. There is also wide variation in the age at which trees flower. In view of this, studies on *Arabidopsis* may not be entirely appropriate for understanding floral development in woody trees. To date, molecular studies on flower development in woody species have been limited to cloning of the floral meristem gene and floral organ identity gene homologues from poplar, pine



and eucalyptus (Tandre et al. 1995; Mouradov et al. 1998; Southerton et al. 1998; Brunner et al. 2000). Moreover, all herbaceous legume models are members of the Papilionoideae, which may be different from tree legumes in their floral development. A better understanding of tree legume floral development at the molecular level may facilitate genetic manipulation of the reproduction process of these species.

In an effort to study floral development of *Acacia mangium*, Wang et al. (2005) constructed a subtractive flower cDNA library against vegetative tissues. A total of 1123 expressed sequence tags (ESTs), representing 576 unique genes, were isolated. Macroarray analysis further identified 147 out of the 576 unique genes expressed specifically during the early, late or whole flowering process. It was found that 10% of these flower-specific genes encoded MADS-domain-containing transcription factors and MYB proteins, 4% other transcription factors, 11% regulatory proteins such as G proteins, kinases and phosphatases, 18% hypothetical proteins and 18% proteins of unknown functions. The isolation of flower-specific transcripts for gibberellic acid (GA) synthesis and GA-induced proteins and other stress- and pathogenesis-related genes (10%) implies their involvement in *Acacia mangium* flower development. The RNA gel blot analysis was shown to correlate well with the results of macroarray analysis. Comparison of the functions of the *Acacia mangium* flower-specific genes and *Arabidopsis* homologues suggested that there was a general conservation of floral development between the two species. Further characterization of the conserved and different flower-specific genes may delineate the flowering process, thereby facilitating genetic modification of the reproduction of this important legume tree.

Plant sterility can be achieved in several ways, such as flower-specific expression of cytotoxic structural genes that can lead to specific ablation of floral organs. Other strategies include sense and antisense gene constructs and RNAi that can specifically suppress transcriptional factors required for floral development. Blocking the reproductive pathway may bring the additional benefit of more rapid vegetative growth through redirecting energy resources to vegetative growth. The isolation and characterization of reproductive development genes from other tree legumes may contribute to and accelerate the production of sterile transgenic trees.

## 7 Legume Nodulation and Nitrogen Fixation

Not all legume species are capable of fixing  $N_2$  in symbiotic relationship with bacteria of the family Rhizobiaceae. In Fabaceae, 97% of the Papilionoideae, 90% of Mimosoideae and only 23% of Caesalpinoideae species can establish a symbiosis, in which the bacteria fix nitrogen within root nodules. Inoculation with rhizobia and mycorrhiza has been shown to enhance the number of nodules, nitrogenase activity and leg-hemoglobin content in plants of *Acacia*

*mangium* and *Mimosa bimucronata* (Patreze and Cordeiro 2004), *Calliandra calothyrsus*, *Gliricidium sepium*, *Leucaena leucocephala* and *Sesbania sesban* (Bala and Giller 2001). Inoculation of in vitro regenerated plants and those introduced from different geographical locations with the most competitive native or in vitro modified rhizobial strain can enhance the growth and survival of legume trees under field conditions. In order to enhance legume nodulation and to modify the host range, host plants can be genetically modified to be more susceptible to *Rhizobium* infection. This was demonstrated in *Lotus*, which after transformation with soybean lectin genes, became susceptible to *Rhizobium japonicum* (Van Rhijn et al. 1998). It has been reported that the expression of opine genes and the malate dehydrogenase (MDH) synthesis gene in roots and nodules can enhance growth of various *Rhizobium* strains (Oger et al. 1997; Miller et al. 1998). Bacterial strains can also be modified to secrete antibiotics, such as trifolitoxin (Robledo et al. 1997), to be more competitive in the growing environment.

## 8 *Acacia mangium* as a Potential Model Plant for Tree Legumes

The genus *Acacia* comprises more than 1300 species, which are representative of legume trees with a wide genetic diversity. The plants are amongst the fastest growing trees on earth. In the genus, *Acacia mangium* is monoecious and flowers at an early age (within 2 years in vegetatively propagated clones). It is a diploid species ( $2n = 2x = 26$ ) with 14–15 linkage groups, and the integrated map spans from 950–1,045 cM (Butcher et al. 2002). Its genome size was estimated to be 1,127 Mbp, which is comparable to that of soybean (Bennett and Leitch 2003). Southern analysis revealed that most tested genes were single copy genes, suggesting a simple genome for *Acacia mangium* (Xie and Hong 2002). Furthermore, *Acacia mangium* possesses great commercial value and is amenable to genetic transformation. All these features are comparable to, or more favorable than, *Populus*, a representative tree genus in the northern hemisphere that is being promoted as a model tree (Taylor 2002). *Acacia mangium* has the potential to serve as a model legume tree species.

## 9 Conclusions

Molecular breeding is a promising approach to crop improvement and it has been used increasingly in agriculture. Public research institutions and biotechnology companies have been generating various novel genetically modified field crops, vegetables and ornamentals, but forest tree species have lagged far behind and, to date, not a single transgenic tree legume has been field tested. Because of increasing demand for wood and its products and diminishing harvestable forests, there is an urgent need for using molecular and

biotechnological tools in forest tree research and improvement. It is anticipated that legume tree species will be a major target for genetic engineering in the 21st century with the availability of improved transformation technology and new cloned genes for different developmental processes. In the near future, trees are expected to be more tolerant to abiotic and biotic stresses, with accelerated growth rate and improved wood structure. In addition, plantation forestry, with optimized and increased productivity, may become the major source of wood products. Accelerated tree improvement programs combining traditional and molecular breeding techniques, together with efficient and cost-effective large-scale clonal propagation of superior clones (e.g. automation of somatic embryo production), are key elements for future reforestation and commercialization of tree legumes.

## References

- Anuradha M, Pullaiah T (1999) In vitro seed culture and induction of enhanced axillary branching in *Pterocarpus santalinus* and *Pterocarpus marsupium*: a method for rapid multiplication. *Phytomorphology* 49:157–163
- Bala A, Giller KE (2001) Symbiotic specificity of tropical tree rhizobia for host legumes. *New Phytol* 149:495–507
- Baweja K, Khurana JP, Khurana P (1995) Influence of light on somatic embryogenesis in hypocotyls of *Albizia lebbek*. *Curr Sci* 68:544–546
- Bennett L (1987) Tissue culturing redbud. *Am Nurseryman* 166:85–91
- Bennett MD, Leitch IJ (2003) Angiosperm DNA C-values database. <http://www.rbgkew.org.uk/cval/homepage.html>
- Bhatnagar S, Kapur A, Khurana P (2004) Evaluation of parameters for high efficiency gene transfer via *Agrobacterium tumefaciens* and production of transformants in Indian mulberry, *Morus indica* cv. K2. *Plant Biotechnol* 21:1–8
- Bon MC, Bonal D, Goh DK, Monteuiis O (1998) Influence of different macronutrient solutions and growth regulators on micropropagation of juvenile *Acacia mangium* and *Paraserianthes falcataria* explants. *Plant Cell Tissue Organ Cult* 53:171–177
- Brunner AM, Rottmann WH, Sheppard LA, Krutovskii K, Di-Fazio SP, Leonardi S, Strauss SH (2000) Structure and expression of duplicate *AGAMOUS* orthologues in poplar. *Plant Mol Biol* 44:619–634
- Burns JA, Wetzstein HY (1998) Embryogenic cultures of the leguminous tree *Albizia julibrissin* and recovery of plants. *Plant Cell Tissue Organ Cult* 54:55–59
- Butcher PA, Moran GF (2000) Genetic linkage mapping in *Acacia mangium*. Development of an integrated map from two outbred pedigrees using RFLP and microsatellite loci. *Theor Appl Genet* 101:594–605
- Butcher PA, Williams ER, Whitaker D, Ling S, Speed TP, Moran GF (2002) Improving linkage analysis in outcrossed forest trees: an example from *Acacia mangium*. *Theor Appl Genet* 104:1185–1191
- Carimi F, Dilenzo R, Crescimanno FG (1997) Callus induction and somatic embryogenesis in carob (*Ceratonia siliqua* L.) from ovule culture. *Sci Hort* 70:73–79
- Caro LA, Polci PA, Lindstrom LI, Echenique CV, Hernandez LF (2002) Micropropagation of *Prosopis chilensis* (Mol) Stuntz from young and mature plants. *Biocell* 26:25–33
- Chalupa V (1983) Micropropagation of conifer and broadleaved forest trees. *Comm Inst For Czechosl* 13:7–39

- Chand S, Singh AK (2004) In vitro shoot regeneration from cotyledonary node explants of a multipurpose leguminous tree, *Pterocarpus marsupium* Roxb. In Vitro Cell Dev Biol-Plant 40:464–466
- Curir P, Ruffoni B, Massabo F, Damiano C (1990) Induction of somatic embryogenesis in *Genista monosperma* Lam. Acta Hort 280:113–116
- Das P, Samantaray S, Roberts AV, Rout GR (1997) In vitro somatic embryogenesis of *Dalbergia sissoo* Roxb: a multipurpose timber-yielding tree. Plant Cell Rep 16:578–582
- Davis JM, Keathley DE (1987) Differential response to in vitro bud culture in mature *Robinia pseudoacacia* L. (black locust). Plant Cell Rep 6:431–434
- De-Meier GC, Bovo OA (2000) Plant regeneration from single nodal stem explant of legume tree *Prosopis alba* Griseb. Biocell 24:89–95
- Detrez C, Ndiaye S, Dreyfus B (1994) In vitro regeneration of the tropical multipurpose leguminous tree *Sesbania grandiflora* from cotyledon explants. Plant Cell Rep 14:87–93
- Dewan A, Nanda K, Gupta SC (1992) In vitro propagation of *Acacia nilotica* subsp. *indica* Brenan via cotyledonary nodes. Plant Cell Rep 12:18–21
- Doyle JJ, Luckow MA (2003) The rest of iceberg. Legume diversity and evolution in a phylogenetic context. Plant Physiol 131:900–910
- Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
- Garg L, Bhandari NN, Rani V, Bhojwani SS (1996) Somatic embryogenesis and regeneration of triploid plants in endosperm cultures of *Acacia nilotica*. Plant Cell Rep 15:855–858
- Geneve RL, Kester ST (1990) The initiation of somatic embryos and adventitious roots from developing zygotic embryo explants of *Cercis canadensis* L. cultured in vitro. Plant Cell Tissue Organ Cult 22:71–76
- George EF (1993) Plant propagation by tissue culture. Part 2: In practice. Exergetics, Edington
- Gharyal PK, Maheshwari SC (1981) In vitro differentiation of somatic embryoids in a leguminous tree *Albizia lebbek* L. Naturwissenschaften 69:379–380
- Gharyal PK, Maheshwari SC (1990) Differentiation in explants from mature leguminous trees. Plant Cell Rep 8:550–553
- Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18:115–135
- Grubisic D, Culafic L (1986) In vitro vegetative propagation of *Cercis siliquastrum* L. Bull Sci Yug A 31:69
- Gulati A, Jaiwal PK (1996) Micropropagation of *Dalbergia sissoo* from nodal explants of mature tree. Biol Plant 38:169–175
- Gutteridge RC, Shelton HM (1994) Forage tree legumes in tropical agriculture. CAB International, Wallingford
- Han KH, Keathley DE (1988) Isolation and culture of protoplasts from callus tissue of black locust (*Robinia pseudoacacia* L.). Nitrogen Fixing Tree Res Rep 6:68–70
- Han KH, Keathley DE, Davis JM, Gordan MP (1993a) Cambial tissue culture and subsequent shoot regeneration from mature black locust (*Robinia pseudoacacia* L.). Plant Cell Rep 12:185–188
- Han KH, Keathley DE, Davis JM, Gordan MP (1993b) Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L., black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. Plant Sci 88:149–157
- Hinchee MAW, Connorward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Bio/Technology 6:915–922
- Huang FH, Al-Khayri JM, Gbur EE (1994) Micropropagation of *Acacia mearnsii*. In Vitro Cell Dev Biol 30:70–74
- Igasaki T, Mohri T, Ichikawa H, Shinohara K (2000) *Agrobacterium tumefaciens*-mediated transformation of *Robinia pseudoacacia*. Plant Cell Rep 19:448–453
- Jones TC, Batchelor CA, Harris PJC (1990) In vitro culture and propagation of *Acacia* species (*A. bivenosa*, *A. holocerecea*, *A. salicina*, *A. saligna* and *A. sclerosperma*). Int Tree Crops J 6:183–192

- Jordan M, Larrain M, Tapia A, Roveraro C (2001) In vitro regeneration of *Sophora toromiro* from seedling explants. *Plant Cell Tissue Organ Cult* 66:89–95
- Kaur K, Kant U (2000) Clonal propagation of *Acacia catechu* Willd by shoot-tip cultures. *Plant Growth Regul* 31:143–145
- Kaur K, Verma B, Kant U (1998) Plants obtained from the Khair tree (*Acacia catechu* Willd) using mature nodal segments. *Plant Cell Rep* 17:427–429
- Khatter S, Mohan Ram HY (1982) Organogenesis in the cultured tissues of *Sesbania sesban*, a leguminous shrub. *Ind J Exp Biol* 20:216–219
- Khurana J, Khurana P (2000) Biolistic-mediated DNA delivery into hypocotyls of a leguminous tree *Albizia lebbbeck* L.: influence of biological and physical parameters. *J Plant Biochem Biotechnol* 9:31–34
- Khurana P, Khurana J (1999) Applications of genetic transformation to tree biotechnology. *Indian J Exp Biol* 37:627–638
- Khurana P, Khurana J, Jani M (2003) Regeneration and genetic transformation of tree legumes with special reference to *Albizia* species. In: Jaiwal PK, Singh RP (eds) *Applied genetics of Leguminosae biotechnology*. Kluwer, Dordrecht, pp 285–300
- Khurana P, Bhatnagar S, Kumari S (2005) Thidiazuron and woody plant tissue culture. *J Plant Biol* 32:1–12
- Kumar S, Agrawal V, Gupta SC (2002) Somatic embryogenesis in the woody legume *Calliandra tweedii*. *Plant Cell Tissue Organ Cult* 71:77–80
- LaxmiSita G, Raghavaswamy BV (1993) Regeneration of plantlets from leaf disc cultures of rose wood: control of leaf abscission and shoot tip necrosis. *Plant Sci* 88:107–112
- Lewis GP, Schrire BD, Mackinder BA, Lock JM (2003) *Legumes of the world*. Royal Botanic Gardens, Kew
- Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* 18:100–127
- Lloyd G, McCown B (1980) Commercially feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. *Proc Int Plant Prop Soc* 30:421–427
- Mackay WA, Tipton JL, Thompson GA (1995) Micropropagation of Mexican redbud, *Cercis canadensis* var. *mexicana*. *Plant Cell Tissue Organ Cult* 43:295–299
- Mehta UJ, Krishnamurthy KV, Hazra S (2000) Regeneration of plants via adventitious bud formation from mature zygotic embryo axis in tamarind (*Tamarindus indica* L). *Curr Sci* 78:1231–1234
- Merkle SA, Wiecko AT (1989) Regeneration of *Robinia pseudoacacia* via somatic embryogenesis. *Can J For Res* 19:285–288
- Miah SAR, Rao R (1996) Regeneration of plantlets from excised roots of *Albizia lebbbeck*. *Ind J Exp Biol* 34:188–189
- Miller SS, Driscoll BT, Gregerson RT, Gnatt JS, Vance CP (1998) Alfalfa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule enhanced MDH. *Plant J* 15:173–184
- Mittal A, Agarwall R, Gupta SC (1989) In vitro development of plantlets from axillary buds of *Acacia auriculiformis*. *Plant Cell Tissue Organ Cult* 9:65–70
- Monteuuis O, Bon MC (2000) Influence of auxins and darkness on in vitro rooting of micro-propagated shoots from mature and juvenile *Acacia mangium*. *Plant Cell Tissue Organ Cult* 63:173–177
- Mouradov A, Glassick T, Hamdorf B, Murphy L, Fowler B, Marla S (1998) *NEEDLY*, a *Pinus radiata* ortholog of *FLORICAULA/LEAFY* genes, expressed in both reproductive and vegetative meristems. *Proc Natl Acad Sci USA* 95:6537–6542
- Murashige T, Skoog F (1962) A revised medium for rapid growth bioassays with tobacco tissue culture. *Physiol Plant* 15:473–497
- Nandwani D, Ramawat KG (1992) High frequency plant regeneration from seedling explants of *Prosopis tamarugo*. *Plant Cell Tissue Organ Cult* 29:173–178
- Nangia S, Singh N (1996) Micropropagation of *Leucaena leucocephala*. *Ann Biol* 12:82–85

- Nguyen NT, Moghaieb REA, Saneoka H, Fujita K (2004) RAPD markers associated with salt tolerance in *Acacia auriculiformis* and *Acacia mangium*. *Plant Sci* 167:797–805
- Oger P, Petit A, Dessaux Y (1997) Genetically engineered plants producing opines alter their biological environment. *Nat Biotechnol* 15:369–372
- Olhoft PM, Lin K, Galbraith J, Nielsen NC, Somers DA (2001) The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary node cells. *Plant Cell Rep* 20:731–737
- Olhoft PM, Flagel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta* 216:723–735
- Patreze CM, Cordeiro L (2004) Nitrogen fixing and vesicular arbuscular mycorrhizal symbioses in tropical legume trees of tribe Mimoseae. *For Eco Manage* 196:275–285
- Pattnaik S, Pradhan C, Naik SK, Chand PK (2000) Shoot organogenesis and plantlet regeneration from hypocotyl-derived cell suspensions of a tree legume, *Dalbergia sissoo* Roxb. *In Vitro Cell Dev Biol- Plant* 36:407–410
- Pradhan C, Kar S, Patnaik S, Chand PK (1998a) Propagation of *Dalbergia sissoo* Roxb. through in vitro shoot proliferation from cotyledonary nodes. *Plant Cell Rep* 18:122–126
- Pradhan C, Pattnaik S, Dwari M, Patnaik SN (1998b) Efficient plant regeneration from cell suspension derived callus of East Indian rose wood (*Dalbergia latifolia* Roxb). *Plant Cell Rep* 18:138–142
- Quoirin M, Galiana A, Goh DKS, Limanton A, Gratio V, Ahee J, Rio M, Oliveira D, Duhoux E, Franche C (2000) Progress towards the genetic transformation of four tropical *Acacia* species: *Acacia mangium*, *Acacia crassiparpa*, *Acacia mearnsii* and *Acacia albida*. In: Mohan Jain S, Minocha SC (eds) *Molecular biology of woody plants*, vol 2. Kluwer, Dordrecht, pp 161–178
- Quoirin M, da Silva MC, Martins KG, de Oliveira DE (2001) Multiplication of juvenile black wattle by microcuttings. *Plant Cell Tissue Organ Cult* 66:199–205
- Quoirin M, Franche C, Koehler H (2002) Transient expression of reporter genes introduced in tissues of two *Acacia* species using a biolistic method. *In Vitro Cell Dev Biol-Plant* 38:487–492
- Rao GVR, Prasad MNV (1991) Plant regeneration from the hypocotyl callus of *Acacia auriculiformis*: multipurpose tree legume. *J Plant Physiol* 137:625–627
- Rao PVL, De DN (1987) Tissue culture propagation of tree legume *Albizia lebbek* (L.) Benth. *Plant Sci* 51:263–267
- Reddy PC, Patil V, Prasad TG, Padma K, Udayakumar M (1995) In vitro axillary bud break and multiple shoot production in *Acacia auriculiformis* by tissue culture technique. *Curr Sci* 69:495–496
- Robledo EA, Scupham AJ, Triplett EW (1997) Trifoliotoxin production in *Rhizobium etli* strain CE3 increases competitiveness for rhizosphere colonization and root nodulation of *Phaseolus vulgaris* in soil. *Mol Plant-Microbe Int* 10:228–233
- Rout GR, Samantaray S, Das P (1995) Somatic embryogenesis and plant regeneration from callus cultures of *Acacia catechu*: a multipurpose leguminous tree. *Plant Cell Tissue Organ Cult* 42:283–285
- Sankhla D, Davis TD, Sankhla N (1996) In vitro regeneration of silk tree (*Albizia julibrissin*). *Plant Cell Tissue Organ Cult* 44:83–86
- Saxena PK, Gill R (1987) Plant regeneration from mesophyll protoplasts of the tree legume *Pithecellobium dulce* Benth. *Plant Sci* 53:257–262
- Schenk RU, Hilderbrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can J Bot* 50:199–204
- Sebastian KT, McComb JA (1986) A micropropagation system for carob (*Ceratonia siliqua* L.). *Sci Hort* 28:127–131
- Shahana S, Gupta SC (2002) Somatic embryogenesis in *Sesbania sesban* var. *bicolor*: a multipurpose fabaceous woody species. *Plant Cell Tissue Organ Cult* 69:289–292
- Simmons MH (1987) *Growing acacias*. Kangaroo Press, Kenthurst
- Singh AK, Chand S, Pattnaik S, Chand PK (2002) Adventitious shoot organogenesis and plant regeneration from cotyledons of *Dalbergia sissoo* Roxb: a timber yielding tree legume. *Plant Cell Tissue Organ Cult* 68:203–209



- Skinner JS, Meilan R, Ma C, Straus SH (2003) The *Populus PTD* promoter imparts floral-predominant expression and enables high levels of floral-organ ablation in *Populus*, *Nicotiana* and *Arabidopsis*. *Mol Breed* 12:119–132
- Skolmen RG (1986) Induction of embryogenesis in *Acacia koa*. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 1. Trees I. Springer, Berlin Heidelberg New York, pp 335–384
- Somers DA, Samac DA, Olhoft PM (2003) Recent advances in legume transformation. *Plant Physiol* 131:892–899
- Sonia PKJ, Gulati A, Dahiya S (1998) Direct organogenesis in hypocotyl cultures of *Tamarindus indica*. *Biol Plant* 41:331–337
- Southerton SG, Marshall H, Mouradov A, Teasdale RD (1998) Eucalypt MADS-box genes expressed in developing flowers. *Plant Physiol* 118:365–372
- Swamy SL, Ganguli JL, Puri S (2004) Regeneration and multiplication of *Albizia procera* Benth through organogenesis. *Agrofor Sys* 60:113–121
- Tandre K, Albert VA, Sundas A, Engstrom P (1995) Conifer homologues to genes that control floral development in angiosperms. *Plant Mol Biol* 27:69–78
- Taylor G (2002) *Populus: Arabidopsis* for Forestry. Do we need a model tree? *Ann Bot* 90:681–689
- Tomar UK, Gupta SC (1988) Somatic embryogenesis and organogenesis in callus cultures of a tree legume: *Albizia richardiana* King. *Plant Cell Rep* 7:70–73
- Toshihiro O, Sonoko T (1999) Plant protoplast isolation from sterile *Acacia mangium* seedlings require  $\beta$ -1,3-glucanase. *J Plant Physiol* 115:110–113
- Trieu AT, Burleigh SH, Kardailsky IV, Maidonado-Mendoza IE, Versaw WK, Baylock LA, Shin H, Chiou IJ, Katagiri H, Dewbre GR, Weigel D, Harrison MJ (2000) Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. *Plant J* 22:531–541
- Trigiano RN, Beaty RM, Graham ET (1988) Somatic embryogenesis from immature embryos of redbud (*Cercis canadensis*). *Plant Cell Rep* 7:148–150
- Van Rhijn P, Goldberg RB, Hirsh AM (1998) *Lotus corniculatus* nodulation specificity is changed by the presence of soybean lectin gene. *Plant Cell* 10:1233–1249
- Vengadesan G, Ganapathi A, Amutha S, Selvaraj N (2002) In vitro propagation of *Acacia* species: a review. *Plant Sci* 163:663–671
- Venketeshwaran S, Gandhi V (1982) Mass propagation and genetic improvement of forest trees for biomass production by tissue culture. *Biomass* 2:5–15
- Vila SK, Rey HY, Mroginiski LA (1999) Micropropagation of *Gleditsia triacanthos* L. (Leguminosae): explants regeneration in vitro. *Phyton Int J Exp Bot* 65:97–102
- Villarreal ML, Rojas G (1996) In vitro propagation of *Mimosa tenuiflora* (Willd) Poir: a Mexican medicinal plant. *Plant Cell Rep* 16:80–82
- Wang XJ, Cao XL, Hong Y (2005) Isolation and characterization of flower specific transcripts in *Acacia mangium*. *Tree Physiol* 25:167–178
- Weaver LA, Trigiano RN (1991) Regeneration of *Cladrastis lutea* (Fabaceae) somatic embryogenesis. *Plant Cell Rep* 10:183–186
- Williams CG, Savolainen O (1996) Inbreeding depression in conifers: implications for using selfing as a breeding strategy. *For Sci* 42:102–117
- Xie DY, Hong Y (2001a) In vitro regeneration of *Acacia mangium* via organogenesis. *Plant Cell Tissue Organ Cult* 66:167–173
- Xie DY, Hong Y (2001b) Regeneration of *Acacia mangium* through somatic embryogenesis. *Plant Cell Rep* 20:34–40
- Xie DY, Hong Y (2002) *Agrobacterium*-mediated genetic transformation of *Acacia mangium*. *Plant Cell Rep* 20:917–922
- Yan-Xiu Z, Dun-Yi Y, Harris PJC (1993) Plant regeneration from callus and explants of *Sesbania* spp. *Plant Cell Tissue Organ Cult* 34:253–260
- Zhou YQ, Zhang GF, Yuan BJ, Chen XC (2001) An efficient protocol for plant regeneration from cotyledons of *Cassia obtusifolia* seedlings. *Israel J Plant Sci* 49:209–212



## II.5 *Casuarina glauca*

M. OBERTELLO<sup>1,2</sup>, C. SANTI<sup>1</sup>, S. SVISTONOFF<sup>1</sup>, V. HOCHER<sup>1</sup>, F. AUGUY<sup>1</sup>,  
L. LAPLAZE<sup>1</sup>, D. BOGUSZ<sup>1</sup>, and C. FRANCHE<sup>1</sup>

### 1 Introduction

*Casuarina glauca* Sieb. ex Spreng is a tropical tree that belongs to the family Casuarinaceae. This family is unique amongst the angiosperms and is assigned to an order of its own, the Casuarinales (Beadle 1981). Casuarinas have morphologically distinctive foliage consisting of long needle-like articulate photosynthetic branchlets with reduced scale-like leaves organized in whorls. The shape of the branchlets reduces the area that is exposed to the air, thereby helping to decrease water loss by transpiration and making this forest tree species well adapted to semi-arid and arid climates.

The natural habitat of *C. glauca* is centered in Australia and consists of a narrow belt extending from Benga in New South Wales to Rock-Hampton in Queensland (National Research Council 1984). It has also been introduced into Israel, Cyprus, India, Kenya, Malawi, South Africa, Egypt, Florida and China. This tall tree can survive on difficult sites where other trees fail because of salinity or high soil moisture. In Israel, *C. glauca* outperforms all the other *Casuarina* species, reaching 20 m after 12–14 years, even on saline water tables.

### 2 Economic Importance

In the field, *C. glauca* bears nitrogen-fixing root nodules induced by the soil actinomycete *Frankia* and is capable of high rates of nitrogen fixation, comparable to those found in legumes. In Egypt, a nitrogen-fixing potential of 288 kg N ha<sup>-1</sup> has been reported for *Casuarina* (Diem and Dommergues 1990). *Casuarina* roots also form an association with mycorrhizal fungi, which facilitates the uptake of minerals such as phosphorus (Diem 1996). As a consequence, these actinorhizal trees are capable of growing in poor and disturbed soils and are important elements in plant communities worldwide. They are used in forestry for timber and pulpwood production; they are also utilized

<sup>1</sup> Laboratoire Rhizogénèse Symbiotique, UMR DIA\_PC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France, e-mail: franche@mpl.ird.fr

<sup>2</sup> Programa de Investigación Sobre Interacciones Biológicas (PIIB), Universidad Nacional de Quilmes, Roque SaenzPena 352, Bernal, (B1876BXD) Buenos Aires, Argentina

in stabilizing desert and coastal dunes, in protecting field crops and in restoring degraded soil sites (Diem and Dommergues 1990). Furthermore, one of the greatest uses of *Casuarina* in the tropics is the production of wood for charcoal. This tree produces fuelwood that has a high calorific value (about 5,000 cal g<sup>-1</sup>); its wood has a low ash content and makes excellent charcoal.

### 3 Transgenic Technology

One major goal of our laboratory work is to study the plant genes that are expressed specifically or at enhanced levels in actinorhizal nodules (Franché et al. 1998). To our knowledge, trees in the Casuarinaceae are the only actinorhizal plants that can be genetically transformed by *Agrobacterium rhizogenes* and *A. tumefaciens* (Diouf et al. 1995; Franché et al. 1997; Smouni et al. 2002). Such transgenic plants are useful systems for exploring the role of the specific proteins involved in the ontogenesis and functioning of the actinorhizal nodule. Besides, transgenic trees have a great potential in applied and environmental areas, including improvement of pathogen and stress tolerance, manipulation of lignin content and composition, improvement of growth and phytoremediation of polluted soils (Herschbach and Kopriva 2002).

#### 3.1 *Agrobacterium rhizogenes*-Mediated Transformation

A rapid procedure for production of transgenic actinorhizal root nodules was first established on *C. glauca* (Diouf et al. 1995). It relies on direct nodulation by *Frankia* of transgenic hairy roots induced by *A. rhizogenes*. Young seedlings of *C. glauca* were wounded on the hypocotyl and inoculated with *A. rhizogenes* A4RS containing the binary vector pBIN35S-*gus-int* that carries the *nptII* gene conferring resistance to kanamycin and a derivative of the  $\beta$ -glucuronidase (GUS) reporter gene expressed only upon transfer to the plant cells and not in *Agrobacterium* (Vancanneyt et al. 1990). After 2 weeks, highly branched roots exhibiting rapid growth were observed at the inoculation site. The normal root system was removed at the stem base, and the composite plant was decontaminated by antibiotics. The fast-growing roots were analyzed for the transfer of the transgenes carried by the plasmid pBIN35S-*gus-int*. After incubation in the GUS substrate X-gluc, blue staining was detected in about 50% of *C. glauca* hairy roots, indicating a co-transfer of the genes carried by the plasmid vector. After inoculation by *Frankia* strain THR (Girgis et al. 1990), nodulation was observed on 40% of the transformed roots, whereas 100% of the non-transformed control plants developed nodules. This decrease in nodulation efficiency has also been observed by Beach and Gresshoff (1988) in *A. rhizogenes*-transformed roots of three forage legumes inoculated with *Rhizobium*, and might be linked to the expression of T-DNA genes originating from the Ri-plasmid. Acetylene-reduction activity was comparable in

transformed and untransformed 4-week-old *Casuarina* nodules. Using this 'composite plant' approach, the expression and the function of a chimeric gene can be studied within 4 months in both roots and nodules of *C. glauca*.

### 3.2 *Agrobacterium tumefaciens*-Mediated Transformation

Several parameters have to be fulfilled in order to regenerate transgenic plants using the natural *A. tumefaciens* gene transfer system (Gelvin 2000). These include (1) the virulence of the *Agrobacterium* strain to permit transfer of the T-DNA into wounded plant cells, (2) efficient selection of transformed cells amongst the population of non-transformed cells, and (3) regeneration of transformed cells into plants.

The natural susceptibility of members of the Casuarinaceae to *A. tumefaciens* has been used to develop a gene transfer procedure. A number of physiological and environmental factors, such as the presence of plant phenolic compounds (acetosyringone), sugars, pH, temperature and osmoprotectant compounds, influence the induction of virulence genes and, consequently, the efficiency of T-DNA transfer from *A. tumefaciens* to the wounded plant cells (Gelvin 2000). In the Casuarinaceae, an optimal number of transgenic calli were obtained when epicotyls were excised from 45-day-old seedlings of *C. glauca*, co-cultivated for 3 days with the *Agrobacterium* strain C58C1 (pGV2260; pBIN35S-*gus-int*) (Vancanneyt et al. 1990) at a pH of 5.6, and subsequently grown in the presence of 50 mg l<sup>-1</sup> kanamycin and 250 mg l<sup>-1</sup> ceftaxime. Within 2 months of culture on nutrient medium containing growth regulators and antibiotics, 26% of the transformed epicotyls from *C. glauca* had developed one to three calli in the presence of kanamycin. Most calli (95%) were shown to express GUS activity. The presence of the transgenes was further confirmed by PCR and Southern blot analyses (Le et al. 1996). Transgenic plants were regenerated in approximately 9 months. The phenotype and the nodulation efficiency by *Frankia* was found to be similar in the transgenic Casuarinaceae and non-transformed control plants, and transgenic nodules were shown to fix nitrogen (Smouni et al. 2002).

This transformation procedure developed for *C. glauca* has two major advantages in that the kanamycin selection is efficient and there are very few escapes. Furthermore, only one medium is required for both bud differentiation and shoot elongation.

## 4 Transgenic Plants Used to Characterize Early Symbiotic Genes

### 4.1 Molecular Studies of Actinorhizal Nodules

Basic knowledge of the symbiotic association between *Frankia* and actinorhizal plants is still lacking. The association shows striking differences to *Rhizo-*

*bium*-legume symbiosis (Pawlowski and Bisseling 1996; Franche et al. 1998; Wall 2000). *Frankia* is a filamentous, branching, Gram-positive actinomycete, whereas Rhizobia are Gram-negative, unicellular bacteria. *Frankia* can interact with a diverse group of dicotyledonous plants, whereas Rhizobia only enter symbiosis with plants from the legume family and with one non-legume, *Parasponia*. Legume root nodules represent stem-like structures with peripheral vascular bundles and infected cells in the central tissue, whereas actinorhizal nodules conserve the structure of a lateral root with a central vascular bundle and peripheral infected cortical tissue (Bogusz et al. 1996).

Understanding of the regulatory events in actinorhizal nodulation at the molecular level is mainly limited to the microsymbiont *Frankia*. This actinomycete is characterized by a slow growth rate and the lack of a genetic transformation system (Simonet et al. 1990). So far, investigations have failed to detect any DNA sequences homologous to the *nod* genes in the *Frankia* genome (C  r  monie et al. 1998). However, in the past decade, some progress has been made in the knowledge of the plant genes that are expressed at different stages of actinorhizal nodule differentiation. Differential screening of nodule cDNA libraries with root and nodule cDNA has resulted in the isolation of a number of nodule-specific or nodule-enhanced plant genes in several actinorhizal plants, including *Alnus*, *Datisca*, *Eleagnus* and *Casuarina* (Pawlowski and Bisseling 1996; Wall 2000; Obertello et al. 2003).

## 4.2 Characterization of *enod40*

*enod40* is an early nodulin gene first isolated from soybean (Yang et al. 1993). In legumes, *enod40* genes are highly conserved and are the key genes for nodule organogenesis and a limiting factor in nodule development (Charon et al. 1999). The *enod40* genes encode transcripts of about 0.7 kb that are characterized by the absence of a long open reading frame (ORF) with two conserved regions, namely, regions I and II (Crespi et al. 1994). A small ORF encoding a peptide of 12 or 13 amino acids has been identified in region I and the translation of an ORF spanning region II has been shown to be necessary for the biological activity of *enod40* (Sousa et al. 2001). In legumes, *enod40* expression is induced at a very early stage by nodulation factors, and is localized in the vascular system of roots, shoots, mature nodules as well as in nodule primordia (Crespi et al. 1994; Charon et al. 1999). The results of a recent study revealed that *enod40* encodes two peptides bound to sucrose synthase, suggesting a role in increasing phloem unloading and/or sink strength determination to induce nodulation (R  hrig et al. 2002).

A homologue of *enod40* was isolated from *C. glauca* (*cgenod40*) (Santi et al. 2003). Sequence comparison with other *enod40* from legumes and non-legumes revealed that in addition to significant similarities, region I was absent from both *C. glauca* and *Alnus glutinosa*, another actinorhizal tree. RNA gel blot analysis revealed a less *cgenod40* expression in actinorhizal nodules than that

observed in legume nodules. In transgenic *C. glauca*, expression of *cgenod40-gus* fusion was detected in the vascular tissue of the roots, shoots and nodules. However, transcription was detected in tissues at the early stages of infection by *Frankia*, including prenodules and nodule primordial, and in response to Nod factors (Santi et al. 2003). These results are different from the scheme described in legumes, suggesting that *enod40* plays a different role in actinorhizal plants. In legumes, phloem unloading is mostly apoplastic in the root nodulation zone, but mostly symplastic (due to a lignified root system) in actinorhizal species, thus explaining why *enod40* is not involved in nodule induction. However, the role of *enod40* in actinorhizal symbiosis is not clear.

#### 4.3 *cg12*, an Early Expressed Symbiotic Gene

*cg12* is an actinorhizal symbiotic gene isolated from *C. glauca* (Laplaze et al. 2000) that encodes a subtilisin-like serine protease (subtilase). Subtilases are a super-family of proteases and are thought to play a role in several different aspects of plant development, including epidermal surface formation, stomatal density and distribution in *Arabidopsis* (Berger and Altmann 2000; Tanaka et al. 2001), response to pathogens (Jorda et al. 1999), lateral root development (Neuteboom et al. 1999) and microsporogenesis (Taylor et al. 1997).

The regulation of *cg12* expression and its possible role during actinorhizal nodule infection has been investigated using the transgenic approach by introducing *cg12* promoter-reporter gene fusions into *C. glauca*. Expression of the reporter gene was observed during the first steps of the infection process, i.e. when *Frankia* was invading deformed root hairs and in root and nodule cortical cells containing growing infection threads. *cg12* expression seems to be correlated with plant cell invasion by the endosymbiont from the start of the symbiotic process (Svistoonoff et al. 2003). It has been suggested that CG12 could participate in cell wall weakening during *Frankia* infection; it might also be involved in the maturation of a polypeptide as part of a signaling cascade in response to infection. Interestingly, the *cg12-gus* construct was found to retain the same expression pattern in transgenic *Medicago truncatula* nodules, indicating that actinorhizal plants and legumes share common mechanisms of transcriptional gene regulation (Svistoonoff et al. 2004).

## 5 Transgenic Casuarinaceae as a Tool for Evolutionary Studies of Symbiotic Genes

Transgenic Casuarinaceae trees are valuable tools to investigate the conservation of the mechanisms for nodule-specific expression between legumes and actinorhizal plants. Using transgenic *Casuarina* expressing *gus* driven by the promoters from early nodulin genes from legumes, transgene expression dur-

ing ontogenesis of the actinorhizal nodules was investigated and the data were compared with those reported in legumes, as discussed below.

### 5.1 Early Nodulin Promoter *Penod12* from *Pisum sativum*

The *enod12* gene encoding a hydroxyproline-rich protein is one of the best characterized early nodulin genes from legumes. Two *enod12* genes, *enod12A* and *enod12B*, have been identified in pea (Govers et al. 1991). These two genes are expressed in roots, in response to inoculation with *Rhizobium* or purified nod factors (Horvath et al. 1993). Expression is found in root hairs of infected pea plants, root cells containing the infection thread, and cortical cells immediately in front of the infection thread. In the mature pea nodule, expression is confined to the distal part of the infection zone, suggesting that ENOD12 is a cell wall protein involved in the infection process (Bauer et al. 1994). To date, in actinorhizal plants, a homologue of this symbiotic gene has not been identified.

The *gus* gene under the control of the promoter from the early pea *Psenod12B* nodulin gene (kindly provided by Dr T. Bisseling, Wageningen Agricultural University, The Netherlands) (Vijn et al. 1995) was introduced into *C. glauca*. The pattern of *Psenod12B-gus* expression was established in transgenic plants regenerated from six transformed calli of *C. glauca*. In nodulated *Casuarinaceae* plants, blue staining was not observed in roots, while in nodules, transgene activity was detected in *Frankia*-infected cells of the nitrogen-fixation zone. A kinetic analysis of GUS activity in *Frankia*-infected roots indicated that the *Psenod12B-gus* construct was not expressed during the early stages of the symbiotic process (unpublished data). Judging from the results of these studies, we conclude that *Psenod12* drives a nodule-specific expression in actinorhizal plants, although an *enod12* homologue has not been detected in *Casuarinaceae*. The specificity of expression conferred by this sequence appears to be different in actinorhizal plants and legumes, whereas *Psenod12* directs expression in the infection zone of leguminous nodules. Preferential expression in the nitrogen-fixation zone in actinorhizae indicates that the signals responsible for early expression are not recognized in this heterologous host plant.

### 5.2 Early Nodulin Promoter *Penod40* from *Glycine max*

The second construct introduced into *Casuarina* consisted of the *gus* gene driven by the promoter *Gmenod40* from the early nodulin gene *enod40* isolated from soybean (Yang et al. 1993). As mentioned previously, *enod40* is expressed specifically at the early stages of the *Rhizobium*-legume interaction, and has a putative role in plant organogenesis. The results of in situ hybridization experiments revealed that in soybean, *Gmenod40* was induced in the first outer cortical cells that were mitotically activated by *Rhizobium* as well as in



the region of the root pericycle facing the nodule primordium. In mature nodules, *enod40* transcripts were detected in the pericycle and vascular bundles, suggesting a role in the late stages of nodule development and/or functioning (Roussis et al. 1995; Vijn et al. 1995). In addition to the expression in symbiotic tissues, *enod40* was also found to be expressed in non-symbiotic organs such as stems, lateral and adventitious root primordia and leaf and stipule primordia (Corich et al. 1998). A number of factors, including purified Nod factors, cytokinin and arbuscular mycorrhiza, have been shown to induce the expression of *enod40* in root tissues (Fang and Hirsch 1998; Mathesius et al. 2000; Staehelin et al. 2001).

Transgenic plants of *C. glauca* expressing the *Gmenod40-gus* construct were obtained following genetic transformation with *Agrobacterium*. Roots and aerial plant parts from 16 transgenic lines were stained for GUS activity. In all plants, GUS activity was associated with the vascular system (Santi et al. 2003). In stems, *Gmenod40-gus* was expressed in the procambium/phloem, whereas in roots expression was found not only in the phloem but also in the xylem parenchyma. Transgene expression was also followed in the course of nodule induction by *Frankia*. In all transgenic lines tested, blue stain was not detected in pre-nodule and nodule primordia. In longitudinal and cross sections of mature actinorhizal nodules, blue staining was visible in the pericycle of nodule vascular bundles, especially at the phloem poles. From these results, we concluded that the signal transduction pathway that leads to the induction of legume *enod40* early in nodule development is not active in actinorhizal plants.

## 6 Gene Transfer Technology for the Improvement of *C. glauca*

The feasibility of genetically engineering *C. glauca* has great promise with respect to the factors that limit classical genetic improvement in forestry, such as the large size of the mature plants and the long sexual generation time. Although genetic engineering in trees is still in its infancy, several studies have clearly established its potential for introducing novel genetic characters, such as herbicide tolerance, insect resistance or the modification of lignin content (Tzfira et al. 1998; Ahuja 2000). Similar strategies could be developed to engineer *Casuarina* trees to resist major pathogens, such as *Rhizoctonia solani*, and insect pests, such as *Lymantria xyliana*.

Understanding the regulation of gene expression is an essential requirement for the success of tree biotechnology. Although the list of transgenic tree species is constantly increasing, most of the reports available on detailed analysis of expression conferred by promoters have been carried out on the genus *Populus* (Jouanin and Pilate 1997; Ahuja 2000). The stability of transgene expression also needs to be assessed over a period of several years because trees are exposed to changing environments.



## 6.1 The 35S Promoter Is Suitable for Expressing Constitutively Useful Genes in *C. glauca*

The cauliflower mosaic virus (CaMV) 35S promoter (Odell et al. 1988) has been the most widely used sequence for the expression of foreign genes into plants and has proved very effective in dicotyledons and in some monocotyledons. The signals regulating the initiation of 35S expression lie within the DNA sequence that extends approximately 400 bp upstream from the start of transcription (Benfey and Chua 1990). Several studies have already demonstrated the ability of the 35S viral promoter to drive gene expression in stably transformed perennials (Ahuja 2000). Experimental data have shown that expression controlled by the 35S promoter is not always constitutive in trees. Differences in the pattern, level and inducibility of transgene expression have been observed between *in vitro*, glasshouse and field environments. Therefore, the expression conferred by the 35S promoter was studied in transgenic *C. glauca* plants that were propagated *in vitro*, followed by growth for 1 year in a glasshouse.

Epicotyls of *C. glauca* were genetically transformed with the disarmed strain of *A. tumefaciens* C58C1(pGV2260; pBIN35S-*gus-int*) (Vancanneyt et al. 1990). From three separate transformation experiments, 62 calli growing on kanamycin were obtained and shoot differentiation occurred within 6–9 months on 21 of these tissues. After incubation with X-gluc, strong blue staining was observed in the aerial part of the transgenic plants. Reporter gene activity generally increased with distance from the shoot apex (Smouni et al. 2002); activity was detected throughout the main root except in the elongation zone. Observations on main root transverse sections showed blue staining to be intense in the vascular system, including the xylem, phloem tissues and pericycle. The large, thin-walled cells of the cortex and rhizoderm were lightly stained. As noted in the aerial parts of the plant, the intensity of staining increased with root diameter and distance from the root tip. In emerging lateral roots, promoter activity was restricted to the root cap. In older lateral roots, blue staining was also visible in the proximal region of the vascular tissue (Smouni et al. 2002).

Twenty transgenic *C. glauca* plants were transferred to a glasshouse and grown for 1 year before further analysis. Histochemical analyses were undertaken of the stem and main root with transverse sections collected every 10 cm along the axis of trees whose average above-ground height was about 60 cm. GUS activity was found mainly in primary and secondary xylem, cambium and secondary phloem of the stem (Smouni et al. 2002). In apical and young zones of the stem, GUS expression was seen at the periphery of stem sections, mostly in the ridges and in the phellogen. In the basal zone of the stem, blue staining was observed in the wood and liber, but was absent in the cork (phellem). In the photosynthetic branchlets of 1-year-old transgenic plants of *C. glauca*, the CaMV 35S promoter was found to confer the same expression pattern as in the *in vitro*-cultivated transgenic trees. In the main root, *gus* expression

was visible in meristematic tissues (cambium and phellogen) and secondary xylem and phloem, but not in the phloem. Variation in GUS activity occurred amongst different lateral roots of the same transformed plant line. Little or no expression was found in the lateral roots at the distal part of the root system, whereas blue staining was seen in young lateral roots beneath the soil surface. However, the heterogeneous pattern of blue staining observed when lateral roots were incubated with X-gluc could be the result of poor penetration by the GUS substrate in lignified intact roots (Smouni et al. 2002).

A detailed fluorometric analysis was undertaken on one transgenic 35S-*gus* *C. glauca* tree (Smouni et al. 2000). GUS-specific activity was found to be 29-fold higher in the root-shoot transition zone than in samples collected from the shoot apex. In lateral branches, the activity was also found to increase from the branch apex to the base; activity in the branch base was systematically lower than in the comparative region of the stem. In the main root, the most *gus* expression was measured in the apex; *gus* reporter gene activity was found globally to be 8-fold lower in the root system as compared to the values obtained for the shoots. In lateral roots, GUS activities were 2.5-fold lower, overall, than the activity levels measured in the same region of the main root.

These data establish that the 35S promoter has a nearly constitutive pattern of expression in transgenic plants of *C. glauca* and remains highly expressed in 1-year-old trees.

## 6.2 Characterization of a Metallothionein Gene from *C. glauca* Involved in Stress Responses

Metallothioneins (MTs) are defined as low molecular weight cysteine-rich proteins that can bind heavy metals and may play a role in their intracellular sequestration and transportation (Robinson et al. 1993). Although their exact function remains unclear, plant MTs are thought to be involved in response to stresses like wounding, pathogen infection and leaf senescence. It has been argued recently that they also function as antioxidants and play a role in plasma membrane repair (Rauser 1999).

A clone for type I MT (*cgMT1*) was isolated from a *C. glauca* nodule cDNA library (Laplaze et al. 2002). In situ hybridization revealed localization of the transcripts in mature *Frankia*-infected cells and in the pericycle. The *gus* gene, under the control of the *cgMT1* promoter, was introduced into *Casuarina* and *Allocasuarina*. In transgenic plants, the *cgMT1* promoter was shown to be primarily active in large *Frankia*-infected cells of the nodule nitrogen-fixing zone, roots and the oldest parts of the shoots. The promoter was found to retain the same specificity of expression in both monocotyledonous and dicotyledonous plants (Ahmadi et al. 2003). Induction experiments performed on transgenic *Arabidopsis* plants carrying the *PcgMT1-gus* construct revealed that the promoter *PcgMT1* responded to wounding, oxidative stress and pathogen infection. Our current hypothesis is that *cgMT1* could be involved in antioxidant

defence against reactive oxygen species induced during the symbiotic process. These results indicate that genes from the actinorhizal tree *Casuarina* can be a source of new promoters and valuable traits that could be introduced for biotechnological purposes into other plant species.

## 7 Conclusions

Two different strategies of gene transfer based on *A. rhizogenes* and *A. tumefaciens* have been developed for *C. glauca*, and the 35S promoter has been shown to be a good candidate for constitutive expression in the genus *Casuarina*. These results pave the way for the introduction of agronomically useful traits into actinorhizal trees, which play an important role in reforestation and soil reclamation in developing countries. Moreover, transgenic trees are a major tool in the study of the regulatory mechanisms that control the expression of the actinorhizal symbiotic genes, in dissecting the roles of specific proteins in nodule development, and in comparing the regulatory mechanisms of legume and actinorhizal symbiotic genes. A deeper understanding of the different types of nitrogen-fixing nodules will help to develop future strategies to modify lateral root development on non-symbiotic plants to enable some to associate with nitrogen-fixing bacteria.

## References

- Ahmadi N, Dellerme S, Laplaze L, Guermache F, Auguy F, Duhoux E, Bogusz D, Guiderdoni E, Franche C (2003) The promoter of a metallothionein-like gene from the tropical tree *Casuarina glauca* is active in both annual dicotyledonous and monocotyledonous plants. *Transgenic Res* 12:271–281
- Ahuja MR (2000) Genetic engineering in forest trees: state of the art and future perspectives. In: Jain SM, Minocha SC (eds) *Molecular biology of woody plants*, vol. 1. Kluwer, Dordrecht, pp 31–49
- Bauer P, Crespi MD, Szecsi J, Allison LA, Schultze M, Ratet P, Kondorosi E, Kondorosi A (1994) Alfalfa *Enod12* genes are differentially regulated during nodule development by Nod factors and *Rhizobium* invasion. *Plant Physiol* 105:585–592
- Beach KH, Gresshoff PM (1988) Characterization and culture of *Agrobacterium rhizogenes* transformed roots of forage legumes. *Plant Sci* 57:73–81
- Beadle NCW (1981) *The vegetation of Australia*. Cambridge University Press, Cambridge
- Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinational regulation of transcription in plants. *Science* 250:959–966
- Berger D, Altmann T (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes Dev* 14:1119–1131
- Bogusz D, Franche C, Gherbi H, Diouf D, Gobé C, Auguy F, Ahée J, Duhoux E (1996) La symbiose *Casuarina*–*Frankia*: approche moléculaire du rôle de la plante-hôte. *Acta Bot Gall* 143:621–635

- C  r  monie H, Cournoyer B, Maillet F, Normand P, Fernandez MP (1998) Genetic complementation of rhizobial *nod* mutants with *Frankia* DNA: artifact or reality? *Mol Gen Genet* 260:115–119
- Charon C, Sousa C, Crespi M, Kondorosi A (1999) Alteration of *enod40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *Plant Cell* 11:1953–1965
- Corich V, Goormachtig S, Lievens S, Van Montagu M, Holsters M (1998) Patterns of *ENOD40* gene expression in stem-borne nodules of *Sesbania rostrata*. *Plant Mol Biol* 37:67–76
- Crespi M, Jurkevitch E, Poir  t M, d'Aubenton-Carafa Y, Petrovics G, Kondorosi E, Kondorosi A (1994) *ENOD40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO J* 13:5099–5112
- Diem HG (1996) Les mycorhizes des plantes actinorhiziennes. *Acta Bot Gall* 143:581–592
- Diem HG, Dommergues YD (1990) Current and potential uses and management of Casuarinaceae in the tropics and subtropics. In: Schwintzer CR, Tjepkema JD (eds) *The biology of Frankia and actinorhizal plants*. Academic Press, New York, pp 317–342
- Diouf D, Gherbi H, Prin Y, Franche C, Duhoux E, Bogusz D (1995) Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree. *Mol Plant Microbe Interact* 8:532–537
- Fang Y, Hirsch A (1998) Studying early nodulin gene *ENOD40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. *Plant Physiol* 116:53–68
- Franch   C, Diouf D, Le QV, N'Diaye A, Gherbi H, Bogusz D, Gob   C, Duhoux E (1997) Genetic transformation of the actinorhizal tree *Allocasuarina verticillata* by *Agrobacterium tumefaciens*. *Plant J* 11:897–904
- Franch   C, Laplace L, Duhoux E, Bogusz D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit Rev Plant Sci* 17:1–28
- Gelvin SB (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu Rev Plant Physiol Plant Mol Biol* 51:223–256
- Girgis ZGM, Ishac ZY, El-Haddad M, Saleh AE, Diem HG, Dommergues RY (1990) First report on isolation and culture of effective *Casuarina*-compatible strains of *Frankia* from Egypt. In: El-Lakany MH, Turnbull JW, Brewbaker JL (eds) *Advances in Casuarina research and utilisation*. Desert Development Center, American University, Cairo, pp 156–164
- Govers F, Harmsen H, Heidstra R, Michielsen P, Prins M, van Kammen A, Bisseling T (1991) Characterization of the pea *ENOD12B* gene and expression analyses of two *ENOD12* genes in nodule, stem and flower tissue. *Mol Gen Genet* 228:160–166
- Herschbach C, Kopriva S (2002) Transgenic trees as tools in tree and plant physiology. *Trees* 16:250–261
- Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Prom   J-C, van Kammen A, Bisseling T (1993) Lipooligosaccharides of *Rhizobium* induce infection related early nodulin gene expression in pea root hairs. *Plant J* 4:727–733
- Jorda L, Coego A, Conejero V, Vera P (1999) A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. *Proc Natl Acad Sci USA* 274:2360–2365
- Jouanin L, Pilate G (1997) Gene expression studies. In: Klopfenstein NB, Chun YW, Kim M-S, Ahuja MR (eds) *Micropropagation, genetic engineering, and molecular biology of Populus*. Gen Tech Rep RM-GTR-297. US Department of Agriculture, Forest Service, Rocky Mount Research Station, Fort Collins, Colorado, pp 65–69
- Laplace L, Ribeiro A, Franche C, Duhoux E, Auguy F, Bogusz D, Pawlowski K (2000) Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homologue of *Alnus glutinosa* *ag12*. *Mol Plant Microbe Interact* 13:113–117
- Laplace L, Gherbi H, Duhoux E, Pawlowski K, Auguy F, Guermache F, Franche C, Bogusz D (2002) Symbiotic and non-symbiotic expression of *cgMT1*, a metallothionein-like gene from the actinorhizal tree *Casuarina glauca*. *Plant Mol Biol* 49:81–92
- Le QV, Bogusz D, Gherbi H, Lappartient A, Duhoux E, Franche C (1996) *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen-fixing tree. *Plant Sci* 118:57–69

- Mathesius U, Charon C, Rolfe B, Kondorosi A, Crespi M (2000) Temporal and spatial order of events during the induction of cortical cell divisions in white clover by *Rhizobium leguminosarum* bv. *trifolii* inoculation or localized cytokinin addition. *Mol Plant Microbe Interact* 13:617–628
- National Research Council (1984) Casuarinas: nitrogen-fixing trees for adverse sites. National Academic Press, Washington DC
- Neuteboom LW, Veth-Tello LM, Clijdesdale OR, Hooykaas PJJ, van der Zall BJ (1999) A novel subtilisin-like protease gene from *Arabidopsis thaliana* is expressed at sites of lateral root emergence. *DNA Res* 26:13–19
- Obertello M, Sy M-O, Laplace L, Santi C, Svistoonoff S, Auguy F, Bogusz D, Franche C (2003) Actinorhizal nitrogen fixing nodules: infection process, molecular biology and genomics. *Afr J Biotechnol* 2:528–538
- Odell JT, Knowlton S, Lin W, Mauvais CJ (1988) Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. *Plant Mol Biol* 10:263–272
- Pawlowski K, Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? *Plant Cell* 6:1899–1913
- Rausser E (1999) Structure and function of metal chelators produced by plants. In: *Cell biochemistry and biophysics*, vol. 31. Humana Press, New Jersey, pp 19–48
- Robinson NJ, Tommey AM, Kuske C, Jackson J (1993) Plant metallothioneins. *Biochem J* 295:1–10
- Röhrig H, Schmidt J, Miklashevichs E, Schell J, John M (2002) Soybean *ENOD40* encodes two peptides that bind to sucrose synthase. *Proc Natl Acad Sci USA* 99:1915–1920
- Roussis A, van de Sande K, Papadopoulos K, Drenth J, Bisseling T, Franssen T, Katinakis P (1995) Characterisation of the soybean gene *GmENOD40-2*. *J Exp Bot* 46:719–724
- Santi C, von Groll U, Chiurazzi M, Auguy F, Bogusz D, Franche C, Pawlowski K (2003) Comparison of nodule induction in legume and actinorhizal symbioses: the induction of actinorhizal nodules does not involve *ENOD40*. *Mol Plant Microbe Interact* 16:808–816
- Simonet P, Normand P, Hirsch M, Akkermans ADL (1990) The genetics of the *Frankia*-actinorhizal symbiosis. In: Gresshoff PM (ed) *Molecular biology of symbiotic nitrogen fixation*. CRC Press, Boca Raton, pp 77–109
- Smouni A, Laplace L, Sy M, Bogusz D, Franche C, Duhoux E (2000) Gene transfer in actinorhizal plants of the family Casuarinaceae. In: Subba Rao NS, Dommergues YR (eds) *Microbial interactions in agriculture and forestry*, vol II. Science Publishers, Enfield, New Hampshire, pp 111–129
- Smouni A, Laplace L, Bogusz D, Guermache F, Auguy F, Duhoux E, Franche C (2002) The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree, *Casuarina glauca*. *Funct Plant Biol* 29:649–656
- Sousa A, Johansson C, Charon C, Manyani H, Saulter C, Kondorosi A, Crespi M (2001) Translational and structural requirements of the early nodulin gene *enod40*, a short open reading frame-containing RNA for elicitation of a cell-specific growth response in the alfalfa root cortex. *Mol Cell Biol* 21:354–366
- Staehelin A, Charon C, Boller T, Crespi M, Kondorosi A (2001) *Medicago truncatula* plants overexpressing the early nodulin gene *enod40* exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules. *Proc Natl Acad Sci USA* 98:15366–15371
- Svistoonoff S, Laplace L, Auguy F, Runions J, Duponnois R, Haseloff J, Franche C, Bogusz D (2003) *cg12* expression is specifically linked to infection of root hairs and cortical cells during *Casuarina glauca* and *Allocauarina verticillata* actinorhizal nodule development. *Mol Plant Microbe Interact* 16:600–607
- Svistoonoff S, Laplace L, Liang J, Ribeiro A, Gouveia MC, Auguy F, Fevereiro P, Franche C, Bogusz D (2004) Infection-related activation of the *cg12* promoter is conserved between actinorhizal and legume-rhizobia root nodule symbioses. *Plant Physiol* 136:3191–3197
- Tanaka H, Onouchi H, Kondo M, Hara-Nishimura I, Nishimura M, Machida C, Machida Y (2001) A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants. *Development* 128:4681–4689
- Taylor AA, Horsch A, Zepczyk A, Hasenkampf CA, Riggs CD (1997) Maturation and secretion of a serine proteinase is associated with events of late microsporogenesis. *Plant J* 12:1261–1271

- Tzfira T, Zuker A, Altman A (1998) Forest-tree biotechnology: genetic transformation and its application to future forests. *TIBTECH* 16:439–446
- Vancanneyt G, Schmidt R, O'Conner-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated plant transformation. *Mol Gen Genet* 220:245–250
- Vijn I, Yang WC, Pallisgard N, Ostergaard Jensen E, van Kammen A, Bisseling T (1995) *VsENOD5*, *VsENOD12* and *VsENOD40* expression during *Rhizobium*-induced nodule formation on *Vicia sativa* roots. *Plant Mol Biol* 28:1111–1119
- Wall LG (2000) The actinorhizal symbiosis. *J Plant Growth Regul* 19:167–182
- Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Bisseling T, Franssen H (1993) Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. *Plant J* 3:573–585

## II.6 Conifers

C. WALTER<sup>1</sup>, M. CARSON<sup>2</sup>, and S. CARSON<sup>2</sup>

### 1 Introduction

The domestication of a relatively small number of plants and animals for the provision of food and making articles for everyday use has contributed significantly to the welfare of mankind. Obviously, many of these domesticated species no longer resemble their original ancestors and they are usually not capable of establishing on their own or without extensive human intervention. For example, the size, shape and productivity of our current-day maize (corn) differ greatly from those of its ancestor, teosinte. Over recent decades, we have witnessed waves of innovation that have led to significantly increased agricultural production and improvement in the quality of products derived from plants and animals. The Green Revolution has enabled us to increase the amount of food produced and to feed more people using the same area of land for production. The use of modern biotechnology, which many have termed the second green revolution, has, in the last 8–10 years, demonstrated a strong capability to produce even more food, more economically, and with a reduced environmental impact (Gianessi et al. 2003; James 2006). It is interesting to note that no other technology has been embraced by farmers at such a rapid pace, even in the face of resistance from influential pressure groups.

This chapter focuses on current applications of biotechnology to conifer forestry, including propagation and cloning, DNA fingerprinting, marker-aided selection (MAS) and the related gene-assisted selection (GAS), and genetic modification (GM). It outlines how modern biotechnologies will, in the near future, provide tools and products that will ensure a sustainable and environmentally acceptable supply of timber and timber products, including the option of not harvesting natural forests.

---

<sup>1</sup> Cellwall Biotechnology Center, SCION – Next Generation Biomaterials, Te Papa Tipu Innovation Park, 49 Sala Street, Private Bag 3020, Rotorua, New Zealand, e-mail: christian.walter@scionresearch.com

<sup>2</sup> Carson Associates Ltd, 34 Parawai Rd, Rotorua 3202, New Zealand



## 2 The Economic Importance of Conifer Plantations

### 2.1 Future Wood Supply and Plantations

Plantation forestry has, during the last 200 years, experienced a transition from the use of forest trees as a wild resource to that of intensive management of forest crops for wood and fiber production (Sedjo 2004). However, even as we enter the early years of the 21st century, it can be said that the predominant public perception of forests (particularly in the northern hemisphere) is that of a natural resource, forming an integral part of the natural environment. Add to this that forest trees are long-term perennials, are often associated with cultural heritage, are largely directed at commodity products, are grown over vast land areas and over periods of 10–80 years, and that their harvesting and management require physical inputs on a very large scale, it is not difficult to understand why new technologies, and particularly new biotechnologies, have as yet been slowly adopted, and have not yet had any real impact on the management of forest crops.

However, plantation forestry is rapidly displacing the harvesting of the world's natural forests. The total world forested area is approximately 3.9 billion ha (Gartland et al. 2002), representing 30% of the land surface area, and the consumption of industrial timber for 2002 exceeded 1.6 billion m<sup>3</sup> globally (Brown and Ball 2000; FAOSTAT interactive database: <http://apps.fao.org/>). Over 40% of the world's fiber supply now comes from the 3% of forests established as plantations (Burley 2002). It has been noted that by 2030, up to 50–75% of the world's industrial wood production will most probably come from planted forests, up from perhaps one-third at the beginning of the 21st century (Sedjo 2004). The emphasis of plantation forestry has shifted from the northern hemisphere to the tropics and subtropics on either side of the equator and even to the warm, temperate climates of New Zealand, Chile and South Africa (Gartland et al. 2002).

### 2.2 Expected World Timber Demand

The FAO (FAOSTAT interactive database: <http://apps.fao.org/>) notes that the consumption of industrial timber for the year 2000 was 1.6–2.2 billion m<sup>3</sup> globally, is growing at an annual rate of approximately 1.7%, and at least as much wood again is being burned for cooking and heating purposes. Most of this wood is being supplied by natural forests, and although the output from plantations is rising rapidly, much of this production is oriented towards supplying the 'value added' paper and wood chip sectors, while the bulk of the global demand is still for structural timbers and fuelwood. Most authors do not expect the global or per capita demand for wood to fall any time in the foreseeable future (Zhu et al. 1999; Fenning and Gershenson 2002).

Actual current timber consumption worldwide is probably in the region of 5–7 billion m<sup>3</sup> year<sup>-1</sup> from 3.2–3.9 billion ha of forested land. It is concerning that the average sustainable yield of timber from forests worldwide may be below 2 m<sup>3</sup> ha<sup>-1</sup> year<sup>-1</sup> (South 1999) and this suggests a global shortfall in the *sustainable* supply of wood, in the order of 1–3 billion m<sup>3</sup> year<sup>-1</sup> at current levels of consumption, even if it were considered acceptable to continue with large-scale harvesting from natural forests.

With the world population expected to rise to nearly 8 billion people by mid-century, it appears certain that demand for wood and fiber products will further increase. The agricultural sector has been able to increase its production without the need to greatly expand the area under cultivation. However, the managed forest sector has yet to replicate this success, and forest plantations will need to increase their productivity relative to natural forests by a factor of 10–50 times. It has been concluded that failing to do so may severely compromise mankind's ability to save natural forests from extinction (Walter and Fenning 2004).

### 2.3 Gains and Financial Returns from Biotechnology-Enhanced Forests

The costs of investments in breeding and biotechnology applications should be considered along with all other investments aimed at achieving positive financial returns from plantations (e.g. Sedjo 2004). This can be done using standard forms of investment analysis, as are widely used in exercises of forest planning and valuation (Casasempere 1984; Liley 2000). Despite their high unit value, even short-rotation plantation trees can require periods from 10–30 years to reach financial maturity. Revenues at rotation end can be high. For example, a typical stand of radiata pine in New Zealand or Chile will yield between 650 and 800 m<sup>3</sup> ha<sup>-1</sup> of wood and fiber by or before age 28, with (in NZ) trees each yielding the forest grower approx. US\$100–140, or between US\$30,000 and US\$40,000 ha<sup>-1</sup> (based on NZFOA statistics). However, since all breeding and propagation costs must be 'carried' by the plantation for many years, there are major constraints on funds expended on R&D investment in new biotechnologies, which may take many additional years to develop to the point of commercialization. Sedjo (2004) lists typical estimated benefits from some biotechnology traits, and shows how traits that impact early in the rotation, such as herbicide resistance, incur a lower interest rate penalty, when compared to the high penalty incurred by rotation-end traits, such as wood and fiber yield. For these latter traits, benefits will need to be very high to justify the required high levels of investment.

In a recent analysis of improvement in tree breeding traits for radiata pine in NZ, Carson (2002) concluded that value gains to the forest grower are low, relative to those that can be achieved further down the value chain. Most benefits accrue to the downstream processors and sellers of wood products, unless growers are provided with incentives in stumpage prices. It is often (but not

always) valid to assume that value gains for different traits will be cumulative. Gains in value from improved log and wood quality can be substantial, and can match or even exceed value gains from increased wood volume. The value of increased fiber yield is low for markets emphasizing the production of timber for export (as for NZ radiata pine), but high when the objective is to increase the production and value of processed products (the predominant situation in South American eucalypts and pines).

In spite of the financial benefits along the value chain being potentially very much higher, Carson (2002) used conservative estimates to conclude that a NZ forest grower can likely afford to pay up to, but no more than, about NZ\$2,000 ha<sup>-1</sup> for the improved planting stock – or up to NZ\$2.00 plant<sup>-1</sup> (assuming a planting rate of 1000 stems ha<sup>-1</sup>). Given that many growers are currently paying up to NZ\$0.45 per plant for the best-available improved crosses, and up to NZ\$1.10 plant<sup>-1</sup> for tested clones, there is not much of a margin for recovering the costs of investment in either tree breeding or new biotechnology applications. Also, biotechnology applications invariably involve much greater investment risk than does conventional tree improvement (Casasempere 2004; Kube and Carson 2004), which has the unfortunate effect of further raising the expectations of return for potential investors. These are largely the technical risks associated with implementing the new, relatively untested technology in large-scale forest plantings. Typically, internal rate of return (IRR) thresholds for investments in tree breeding and other research set by government agencies are in the 3–4% range (Casasempere 2004), whereas forest growers using the improved genotypes typically expect IRR in the order of 8–12% (Liley 2000). In contrast, venture capitalists investing in R&D aimed at commercializing biotechnology applications will routinely expect an IRR of up to, or in excess of, 25–35% (A. Casasempere, personal communication). For new biotechnology applications, this suggests that only those applications that are considered ready for application will attract investment at low IRR. R&D funding during the commercialization phase of new biotechnologies will require high IRR, in turn implying that either R&D costs will need to be low, or benefits will need to be either high and/or will need to be allocated over a large total estate, and/or will need to be in the form of reduced costs early in the forest rotation.

The costs of commercializing R&D are usually high. For example, the cost incurred by the New Zealand Forest Research Institute in tree breeding for the 30 years between 1959 and 1989 was an estimated NZ\$30M (US\$20M) in 1989 dollars (J. Buddle, personal communication), while costs of development of several competing somatic embryogenesis (SE) cloning systems for pines over a 10- to 15-year period have been estimated in the range of US\$10–30M (Carson, unpublished data). Despite these high R&D costs, benefit/cost estimates for tree breeding have been shown to be large and positive, e.g. in excess of 48:1 (Shelbourne et al. 1989), largely due to the multiplier effect associated with broad deployment of improved genotypes in plantations. A similar multiplier effect will need to apply to successful biotechnology applications.

What are the implications of these high financial hurdles to the successful implementation of forest biotechnologies? Perhaps government agencies in some countries will continue to invest in 'start-up' grant and loan schemes, with low expectations of return. However, it seems most likely that commercial business must eventually play the most important role, and that new applications of biotechnology will need to be examined in a similar manner to any new business investment, including the development of a business plan, with a strong technical strategy, and both benefits and risks clearly identified. Potential investors will require this as a starting point, and they will then concern themselves with issues of intellectual property (IP) and business structures.

### **3 State of Current Research, Including Tissue Culture, Propagation, Molecular Genetics and Genetic Engineering**

#### **3.1 Modern Biotechnologies for Plantation Forests**

New biotechnologies are based on a better understanding of the information held in the genome, and the resulting ability to modify and control bioprocesses. Considerable success is being reported in these areas, with cloning, and DNA marker and genetic modification (GM) applications are now being used successfully in commercially viable agricultural crops. By May 2005, a total of more than 1 billion acres of genetically modified crops had been planted over the last 10 years (Anon 2005; James 2006) and the commercial, environmental and health benefits have been widely studied and reviewed (Shintani and DellaPenna 1998; Guerinot 2000; Phipps and Park 2002; Pray et al. 2002; Vasil 2003; Ammann 2004). The uptake of these new technologies by farmers and producers has been exceptionally swift, in spite of the considerable impact of scare campaigns launched by environmental pressure groups (Taverne 2005).

However, we now must ask, "has there been similar progress in the forestry sector, and can similar gains be achieved?". Gartland et al. (2002) noted that forest biotechnology research has been grouped around three main themes: (1) improving product quantity and quality, (2) forest genomics and proteomics, and (3) understanding the biotic and abiotic stress responses of trees. These topics are clearly both strategic and important, but only the first, 'improving product quantity and quality', seems designed to meet the technical challenges and goals that will bring short-term benefits to the plantation forestry sector.

Numerous biotechnologies intended for plantation forestry are already beyond the 'proof of concept' stage, yet they may involve further R&D before commercial deployment, which needs to be equally rigorous and many times more expensive. However, there appears often to be a marked absence of both

adequate commercial structures that will attract the necessary investment, and the rewards (not only financial) that will motivate top forestry scientists to work on R&D implementation. Also, the adoption of GM in forestry will require a significant shift in current public thinking. Clearly, some of these topics will require the development of appropriate social, regulatory and commercial policies and structures before they can be successfully implemented.

### 3.2 Conventional Tree Improvement

Conifer breeding began in the southern US (Texas and North Carolina) in the early 1950s, and was quickly followed by programs elsewhere in the US, and in New Zealand, South Africa, South America and Australia (Zobel and Talbert 1984). This has proven to be a very effective tool, and since the level of domestication of forest trees is still very low when compared to agricultural crops, future breeding is expected to achieve substantially increased genetic gains. Many programs report yield increases in the order of 1% or more for each year of tree improvement effort, and with similar levels of gains in other traits (Sedjo 2004; White and Carson 2004). Tree breeders now require new tools that can be integrated with conventional methods to confirm identity and pedigree, enable more efficient selection of progenies and clones, assist understanding of gene function, and the genetic architecture of populations, alter and add to the genetic code itself, by transferring new traits into breeding populations, and influence gene expression.

Tree breeding can only be effective when intensive selection is applied in breeding populations to identify relatively few genotypes (that is, families and/or clones) according to a well-defined breeding goal (Borralho 2001). These genotypes then need to be massively multiplied for plantation establishment. Thus, the search for increasingly more cost-effective propagation methods becomes imperative, and this need is greatest for those programs aiming to achieve gains from either family or (particularly) clonal forestry. Effective propagation technologies are usually also a prerequisite for the successful application of modern gene technologies to forestry (Walter et al. 1998, 1999; Klimaszewska et al. 2003; Tang and Newton 2003).

The first, open-pollinated seed orchards of conifers and many hardwoods, while effective in delivering substantial genetic gains, were relatively inefficient (Sweet 1992). The solution developed subsequently for most major plantation pines, and some hardwoods, was to develop 'control-pollinated' (or CP) seed orchards, and these orchards are now the industry standard (Carson et al. 1992). In addition, vegetative propagation methods have been applied successfully to most conifers, and notably for the major pine species used in southern hemisphere countries (that is, *P. taeda*, *P. radiata*, *P. elliottii*, and *P. caribaea* × *P. elliottii*) (Haines and Walker 1996; Balocchi 1997; Menzies and Aimers-Halliday 2004).

### 3.3 Clonal Forestry

Clonal forestry, as a form of plantation forestry, can be defined as afforestation with a restricted number of vegetatively propagated clones, which have been tested and selected in clonal tests, the best being subsequently mass produced (Menzies and Aimers-Halliday 2004). Although implementation has been slow with conifers, there are numerous successful eucalypt clonal forestry programs, some of which have been active for many years (Zobel 1993; Griffin et al. 2000). The potential benefits of clonal forestry for pines have often been cited (Libby 1982; Libby and Rauter 1984; Carson 1986) including gains arising from testing and selection of clones, clone/site matching to increase genetic gains from both capture of favorable genotype by environment effects ( $G \times E$ ) and targeting expression to existing site properties, greater uniformity, which may have little impact on growth and yield traits, but can be extremely valuable for log and wood quality and disease resistance traits, and for harvesting and processing, and greater repeatability, which provides benefits in yield prediction and planning.

Clonal forestry has been criticized for its perceived potential to reduce biodiversity, and there are a number of risk factors that need to be addressed (Kube and Carson 2004). Principal among these is the potential risk of 'genetic monocultures' from damaging losses due to pests and diseases. However, this risk is being defined as largely being a function of sample size, in which anywhere between about 15 and 120 clones may be deemed sufficient to manage it (Libby 1982; Hühn 1992; Bishir and Roberds 1999; Kube and Carson 2004). It is also instructive to consider the beneficial effect of producing wood in clonal forests at high efficiency as compared to natural stands. Wood produced in this way offers the potential to substitute for a significant portion of the harvest currently coming from native forests, thereby enhancing biodiversity.

Clonal programs in conifers have combined the use of tissue culture with further multiplication using rooted cuttings to provide a cost-effective route to forestry (Libby and Hood 1976; Menzies 1994; Haines and Walker 1996; Menzies and Aimers-Halliday 1997). SE can also be used either as an *in vitro* clonal storage method only, and/or for clonal forestry (Smith et al. 1994; Menzies and Aimers-Halliday 2004; Sutton et al. 2004).

The main short-term technical challenges for implementing clonal forestry are to (1) develop and extend cost-efficient cuttings methods to more species, species hybrids and genotypes, (2) develop more cost-efficient tissue culture systems to enable rapid bulking-up of selected clonal stocks, while retaining juvenility (e.g. Hargreaves and Smith 1994a, b), and (3) develop cost-effective artificial seed systems (Sutton et al. 2004).

At current rates of R&D progress, there can be little doubt that clonal forestry will become increasingly widespread in the near future in response to an increasing world demand for sustainably managed wood and fiber products.



### 3.4 DNA Markers

DNA markers are just beginning to have a major impact in forest tree improvement programs. DNA fingerprinting is being used routinely as a tool for quality control (Wilcox et al. 1997), for example in studying the genetic diversity of breeding population accessions from native provenance and land-race origins, verifying the genetic identity of plus-tree candidates held in clonal archives, paternity testing of progeny in screening trials, and verifying the genetic identity of seed orchard parents and production clones in tissue culture operations during stages of multiplication of elite stock for deployment.

Marker-aided selection (MAS) is being used extensively in agricultural crops. Although MAS has not been implemented in trees, it promises to be a powerful tool for obtaining genetic gains through bypassing the need for long-term field trials and shortening the time required for selection. MAS could now be applied directly for early screening of progenies and clones of radiata pine (Devey et al. 2003; Carson et al., unpublished data). Initial costs of the research are high, but can be justified on the basis of benefit/cost calculations (Wilcox et al. 2001). Project costs can be expected to reduce substantially as the costs of genotyping are reduced by the use of new technologies. High-throughput laboratories are being established to carry out this work as a service. DNA genotyping will, in turn, open up opportunities for extending genetic quality control by making it economically viable to use fingerprinting to track products along the forestry value chain. Expected advances in technology, such as microchips and the use of differential displays, could soon allow rapid and cost-efficient screening of DNA samples, thus significantly lowering the cost thresholds for MAS and DNA fingerprinting.

There are significant short-term technical challenges in implementing DNA marker-based applications. These include the need to improve marker systems, and reduce processing costs, implement and improve MAS and GAS methods, and establish more 'gene mapping trials' for QTL detection.

### 3.5 Genetic Engineering

Many plant species, in particular those of relevance to agriculture, have been genetically modified, including cotton, maize, potato, tomato, oilseed rape and alfalfa (An et al. 1986; Deak et al. 1986; Shahin et al. 1986; Pua et al. 1987). Techniques include using *Agrobacterium* as a natural gene transfer vector, and also a range of methods to directly introduce pure DNA into cells, with subsequent integration into the genome (Fromm et al. 1986; Spangenberg et al. 1986; Klein et al. 1987). The new information can lead to the expression of a trait that is new to the engineered species, and beneficial in a commercial or environmental context.

Forest biotechnology is lagging behind and, to date, the only report of commercial plantations with genetically modified trees is for poplar in China



(Xhiao-hua et al. 2003). However, most commercially relevant tree species have been transformed, and results have demonstrated the correct and continued expression of new genes in these plants (Fillatti et al. 1987; Walter et al. 1998, 1999; Bishop-Hurley et al. 2001; Grace et al. 2005). Considerable numbers of transgenic trees have been under recent regulatory examination in the US, indicating a high level of current interest in commercializing these applications (Sedjo 2004; FAO 2005).

In many cases, the introduced traits are identical to those used in agriculture, but this may change when traits become available that will allow the targeted modification of particular wood-related end uses, for example, those associated with the secondary cell wall. It is also important to note that trees can, besides producing raw materials for the timber and pulp and paper industry, provide resources for novel biomaterials such as composites, fine chemicals, pharmaceuticals and bioenergy resources, and research is aimed at improving trees for such use.

The most important benefits to tree plantations of GM can arise in particular from the transfer of traits that are not readily available either in the breeding population or the genetic resource. Some examples are branch size and angle, stem shape (taper and 'roundness') and traits such as lignin content/composition, and herbicide, fungal and insect resistance (e.g. Shin et al. 1994; Punja 2001; Bishop-Hurley et al. 2001; Li et al. 2003; Tang and Tian 2003; Sedjo 2004; Charity et al. 2005; Grace et al. 2005). The potential environmental benefit of such technology, as demonstrated, for example, in agricultural applications (Gianessi et al. 2002), lies with the reduced amounts of toxic chemicals required to fight pest outbreaks, and with the production of additional timber that would otherwise have to be resourced from native forests, which these now protected forests can provide. Further, the considerable amount of toxic waste currently produced in pulp production can be significantly reduced by engineering lignin biochemistry, and making the extraction of cellulose for the production of paper less toxic.

Tree genetic engineering significantly lags behind the commercial successes demonstrated in agriculture. Currently, genetically modified trees are assessed for the expression of their new traits in the glasshouse and in some field trials of limited size (for a discussion of the situation in Europe see Fenning and Gershenzon 2003; a comprehensive overview of field testing for genetically engineered trees is given in FAO 2005).

Gene transfer technologies will initially be very expensive to apply, and, as discussed later, their acceptance will require initial reassurances that public safety and environmental protection are not placed at risk. It will be important to follow closely the discussion concerning the safety of GM crops and results from risk assessment studies, and to relate these to the benefits of GM trees for the forest grower, the manufacturer and the environment. It is also imperative that regulatory agencies consider risk and benefit related to GM trees on the basis of scientific evidence, rather than yielding to political pressure and emotional argument in their decision making.

The main technical barriers needing to be addressed in the 5- to 10-year term are to (1) improve and extend the efficiency of gene transfer methods, including reducing costs/improving transformation efficiencies/extending to more genotypes, ensuring long-term fidelity of novel gene expression, and improving techniques to reduce the impacts of gene silencing, (2) develop GM methods that will win greater public acceptance alongside better educational efforts, and (3) develop techniques that increase the genetic variation available to selection (that is, the concept of transferring more than one or two genes, perhaps even a large part of a chromosome, and then using conventional selection on the resulting transformed clones).

## 4 Potential Applications for Transgenic Conifers in Plantation Forestry

By way of example, some applications of biotechnology and genetic engineering are discussed below that have been developed over the last few years and that may actually provide the first wave of genetically modified conifer trees for plantation forestry.

### 4.1 Insect Resistance

Similar to many agricultural crops, trees are subject to pressure from various disease and insect pests. Significant economic damage is done by lepidopteran insects such as the diamondback moth or the Asian gypsy moth. Fortunately, a considerable body of data has been collected over the last 10 years on the efficiency of insect resistance genes expressed in agricultural crops, and in particular a gene from the bacterium *Bacillus thuringiensis*, the Bt toxin gene. A range of Bt genes are available and they are characterized by high toxicity to and high specificity for certain lepidopteran insects, and absence of toxicity to other organisms. Crops genetically engineered with these genes have allowed farmers to produce more on less land, at the same time increasing profit and significantly reducing the frequency of pesticide applications (Pray et al. 2002). This practise further improves the economics and provides for substantial environmental benefit. It is important to note in our discussion on public perception that a mixture of several Bt toxins is actually approved for organic farming, whereas only one or, more recently, two specific Bt proteins are expressed in genetically modified plants. In forestry, Bt-engineered poplar is now present in commercial plantations in China (Xhiao-hua et al. 2003; FAO 2005). The environmental benefit is mainly seen in the ability to avoid losses through pest infestations, thereby producing greater amounts of valuable timber, the balance of which can reduce harvests in natural forests.

*Pinus radiata*, one of the important conifer plantation species, has been engineered with a Bt gene and studies have confirmed resistance of engineered

trees against a lepidopteran insect (Grace et al. 2005). It is possible that Bt-engineered *Pinus radiata* will be the first genetically engineered conifer for commercial use.

## 4.2 Modification of Lignin and Cellulose Biochemistry

The pulp and paper industry is one of the biggest industries worldwide. Most raw materials for making paper are based on wood (with a minor amount of recyclable material which is also mainly based on wood), and the major component of paper, cellulose, has to be extracted from wood in a process that removes lignin. This creates large amounts of waste materials in a process that is environmentally problematic. Novel strategies that are particularly relevant to conifer forestry have been designed to change lignin biochemistry in conifers to make lignins more extractable and the process less harmful to the environment (Pilate et al. 2002; Li et al. 2003). In short, this is achieved by a genetic engineering approach which modifies conifer lignin such that it resembles hardwood lignin, which is easier to extract and leaves less toxic waste. Importantly, this technology does not reduce the amount of lignin, which might otherwise have undesirable effects on the stability of the tree or its ability to fend off various pests and diseases. Field testing of transgenic poplar engineered for modified lignin has resulted in encouraging results as to the extractability of modified lignin and the continued ability of the trees to grow and perform similarly to untransformed controls (Pilate et al. 2002). Field tests with lignin-modified conifers are in progress (FAO 2005).

## 4.3 Herbicide Resistance

Herbicides are not commonly used in mature plantation forests and a major need is not foreseen for herbicide applications, since well-established trees are able to protect themselves from competing weeds. The situation is different, however, in the nursery and in the first years of establishment of young trees in the forests. Here, weed competition is a major management challenge for foresters and nursery staff. Also, some areas are uneconomic for planting, owing to a heavy weed load. Conifers engineered for environmentally benign herbicides have been produced (Bishop-Hurley et al. 2001; Charity et al. 2005) and have been shown to thrive under the application of herbicides that will eradicate competing weed species. While herbicide resistance is not envisaged as playing a major role in established plantations, this trait is easily and economically engineered and has the added advantage that any changes of gene expression late in development of the trees will have few negative effects, simply because the trait is no longer required. Further, the use of engineered trees in conjunction with benign herbicides allows the phasing out of more toxic chemicals that are currently in use in nurseries and young plantations.

#### 4.4 Durability

Timber from conifer trees usually lacks natural durability, and this is of great concern for the building industry, particularly where structural timber comes in contact with trapped water and soil moisture. For example, *Pinus radiata* timber is frequently treated with various chemicals for durability, including copper-chrome-arsenate (CCA) treatment for outdoor applications. Although this method is very efficient, CCA is becoming less acceptable with consumers and some uses of CCA-treated radiata pine have been banned in Australia. Alternatives must be found and it is conceivable that genes that provide durability in other tree species may be a good resource for genetic engineering towards higher durability in pines. For example, the New Zealand totara tree (*Podocarpus totara*) produces highly durable wood due to the production and deposition in wood cells of the chemical totarol (amongst other compounds that enhance durability). The biochemical pathway leading to totarol production is known and the respective genes may well provide a suitable resource for durability in genetically engineered conifer trees.

#### 4.5 New Biomaterials Produced by Trees

In recent years, a considerable body of information has been generated on the genetic information stored in various organisms, including, for example, humans, rice, poplar and, to some extent, conifers. Databases that store gene and cDNA sequences are mainly publicly available [through the National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pubmed>], and this information provides the tools to better understand and eventually modify biochemical pathways in agricultural crops and forest trees. New secondary metabolites can be made in commercially important plant species and this will lead to new raw materials (biomaterials) that can provide the source for pharmaceuticals, fine chemicals, biofuels and composites.

#### 4.6 A Vision for Future Forests

Although the available tools of biotechnology are already finding uses in current forest tree improvement and plantation forestry, what will be the future of plantation forestry, and what new and valuable applications need now to be identified?

Based on current trends in plantation forestry, it seems likely that plantation forestry will become more mechanized and more intensively managed than it is today. Log and wood quality will become increasingly important. There will be an increasing trend towards the separation of the forest growing business from that of processing and production. This has already become evident in the US, Australia and New Zealand with forest ownership by Real Estate Investment

Trusts and Timber Investment Management Organizations. Investors will demand that the higher-quality wood be produced in short forest crop rotations, with decreased growing costs, and with much more efficient use of land. Forest growers will increasingly target niche product markets from differentiated purpose-grown stands. It will become more and more important to better predict yields of wood products, and to be able to better segregate logs, and parts of logs, into the optimum set of products, as soon as possible after harvest. The new forest crops will need to produce high yields on lands considered marginal for growing food crops, and will increasingly need to provide their own nutritional needs, without fertilizer inputs, and to be less demanding on water supplies. They will also need to be more tolerant of limitations imposed by salt and aluminum levels in the soil, and to be able to withstand attack by a more diverse range of insects and pathogens. Demand for wood and wood products will increase significantly with the increase in world population numbers and higher standards of living, in particular in developing countries. The public will demand environmentally sustainable wood production and this will lead to strong support for the productivity of plantation forestry to be enhanced using modern biotechnologies. Pressures on natural forests will increase, and the insight that unsustainable harvesting practises must not be continued will lead to even stronger support for applications of biotechnology. In many regions, the community will demand that wood be produced without the use of herbicides, pesticides and other synthetic chemicals. Trees will sometimes be required to act as 'factories' for new biomaterials and types of fibers, as well as for timber. Wood products will not necessarily come from forests alone, but potentially from bioreactor-supported production that can react flexibly to client demands. These facilities will produce specialized new raw materials that may replace other currently used products (for example, aluminum in the aviation industry).

There are abundant challenges for biotechnology in this view of the future. To progress them, tree breeders and biotechnologists will need to be increasingly aware of the global context within which they work, and the broader challenges they face in achieving public acceptance. Successful implementation of new biotechnologies in any field will also require a more balanced and fact-based public discussion of risk, in particular the risks of not embracing this new technology. This leads us to offer a few thoughts on the social challenges related to forest biotechnology.

## **5 Social Challenges**

### **5.1 Public Concerns with Genetic Engineering**

While agricultural biotechnology continues to have a significant impact (James 2006; ISAAA, [www.isaaa.org](http://www.isaaa.org)), very strong opposition is being voiced concern-

ing the use of genetically modified plants, including non-food crops such as cotton and trees. Concerns have been raised from economic, environmental, health and social/cultural perspectives (Dale 1999; Owusu 1999; Stewart et al. 2000; Thompson Campbell 2000; Quist and Chapela 2001; Dale et al. 2002; Burdon and Walter 2004; Taverne 2005). However, it is increasingly obvious that, in general, farmers' profits have increased, while at the same time the use of environmentally threatening pesticides has been significantly reduced in many crops (Gianessi et al. 2002, 2003; Phipps and Park 2002; Demont and Tollens 2004; James 2006). Also, there have been reports of reduced health problems due to reductions in pesticide use, and amongst those applying the chemicals (Pray et al. 2002; Hossain et al. 2004). More recently, genetically engineered crops have been designed that will make significant contributions to human health. Golden rice fortified with increased levels of vitamin A provides a very good example (Guerinot 2000; Paine et al. 2005). The continued resistance by certain environmental groups to the implementation of such crops is seen by some as a crime against humanity (Moore 2004). It has also been argued that the current risk-averse position of our society in general is based on a deeply held, pessimistic view of what science can contribute to human welfare, and a resulting inability to balance risk and benefit (Taverne 2005).

Given these issues surrounding genetic engineering in agriculture, it is important to assess whether similar risks and benefits result from this technology when applied to plantation forestry. Misconceptions and inconsistent attitudes and approaches to risks, especially those associated with new technologies, can hinder the application of forest biotechnology and deprive us of powerful solutions to pressing environmental and economic problems.

In the debate on genetic engineering, concerns are sometimes expressed that something unexpected will happen with GM plants, and this is seen as an unacceptable risk. However, this unexpected effect is usually not defined, nor is its occurrence substantiated in any way. It is argued instead that because of the unexpected element, the risk is too high and GM should not proceed. The precautionary principle is frequently used to support this argument. It can, however, be argued that, in human society, any activity is associated with some degree of risk. A risk-free environment is impossible to achieve, and humans make many subconscious and conscious decisions whether or not to accept a specific risk in order to gain some benefit. In the discussions surrounding genetic engineering, it often appears as if the risk associated with this new technology is being compared to a situation where risk does not exist. Consequently, the taking of any risk, regardless of benefit, is seen as an unacceptable option, and so it is asserted that all use of GM should be terminated. The precautionary principle is again employed to support a stance of 'do nothing when in doubt'. Proponents of genetic engineering technology argue that this approach is flawed, and that the risk associated with a specific outcome (the environmentally sustainable production of wood, for example) must be compared to *how else* this particular outcome may be achieved, rather

than 'doing nothing'. They feel that interpreting the precautionary principle in the way described also amounts to a decision, with its own consequences, intended or otherwise (Conner et al. 2003), and that the results could well be worse than the risk related to the dismissed technology. Interestingly, some authors argue that the precautionary principle can actually be applied to halt all resistance against GM (Van den Belt 2003; Walter 2004). This also illustrates that a principle that has been designed with the best intentions can actually become a serious threat to scientific and social progress, by effectively halting or delaying any new technology that has potential to solve environmental or economic problems (Taverne 2005).

Further, we note that despite claims that GM is imprecise and risky, existing and often widely accepted technologies, such as conventional tree breeding, are themselves not risk-free (e.g. Kube and Carson 2004). Traditional breeding is an intrinsically imprecise process with regard to both numbers and types of genes recombined and the new variation that may be created, either through recombination or through the appearance of mutations. Breeders use selection in an attempt to remove much unwanted variation, but it is still possible for mistakes to occur, and many changes certainly go unnoticed. This appears to be much less controllable than genetic engineering, where only one or a few characterized genes may be either added or their expression altered (Conner et al. 2003).

We note also that many risk issues discussed in relation to genetic engineering are not intrinsic to this technology (or technology inherent). Conversely, the implied consequences of using it could also occur without using it [this is known as technology transcending (Leisinger 1996)]. Finally, the risk of using genetic engineering for tree improvement must be discussed in comparison with the risk of not using this technology; that is, what options for solving environmental, social or economic problems are we denying ourselves by not using genetic engineering in forestry (Gartland et al. 2002)?

## 5.2 Risks to the Environment and Human Health

As we have mentioned previously, plantation forest tree species are long-lived perennials for which breeding programs have only advanced a few generations from their wild native forebears. Also, while many of these species (particularly in the southern hemisphere) are grown in exotic locations, and among unrelated tree species, many also are grown in their area of native origin (or 'provenance') in intimate association with their close wild relatives. Not surprisingly, these factors can lead to heightened public concerns about any negative impacts to the environment that may arise from applications of biotechnology to trees.



### 5.3 Fidelity of Gene Expression

The long-term stability of expression of introduced genes will be a critical feature for long-rotation plantation trees. Genes introduced into the host organism are subject to a variety of mechanisms which can all lead to undesirable changes in gene expression during the organism's life span (Matzke and Matzke 1995; Meyer 1995; Kumar and Fladung 2001). Although this risk of altered gene expression may always be present, its likelihood can be minimized and its effects controlled. For example, recent research results indicate that instability of gene expression in a given transgenic tree usually becomes obvious at early stages of tree development (that is, in the nursery), which thus provides opportunities to cull undesired events before they enter the forest (Walter 2004; Walter, unpublished data).

The deployment of a population of trees containing multiple independent transgenic events is a further strategy to mitigate risk. This is already practised in clonal forestry (by deployment of multiple clones), and should one event fail, the risk of losing the entire plantation is still contained (Burdon 2001).

A further concern relates to the long-term efficiency of the introduced gene construct. For example, while a resistance gene against an insect may continue to be correctly expressed over the rotation, the insect populations may become resistant, rendering the gene inefficient. Appropriate risk management and/or mitigation strategies may include (the favored approach of) 'pyramiding' of several resistance genes acting on different parts of the insect life cycle, and/or including some sensitive non-GM trees within the plantation to reduce the selection pressure on the pest so as to overcome the resistance marker (Krattiger 1997; Nester et al. 2002). However, it is interesting to note that, despite the extensive use of the *Bacillus thuringiensis* (Bt) gene in transgenic crops, this has not yet resulted in the development of Bt insect-resistant populations (Fox 2003).

### 5.4 Gene Movement Between Species: Horizontal Gene Transfer

Horizontal gene transfer (HGT) has attracted much scientific attention and is an integral part of the public debate on GM (Snow 2002; Tepfer et al. 2003). It is regarded as a mechanism supporting the evolution of species (Jain et al. 1999; De la Cruz and Davies 2000; Ochmann et al. 2000), and is a natural process where genetic material (DNA) is transferred from one organism into another, sometimes very distantly related organism by non-sexual means. Several examples of such gene transfer are well known (Binns and Thomashow 1988; Lorenz and Wackernagel 1994; Droege et al. 1999; De Vries et al. 2001).

However, while the transfer of DNA within bacterial species has been demonstrated in nature, other HGT events have proven elusive to demonstrate (Nielsen et al. 1998; Conner et al. 2003; Tepfer et al. 2003). Many laboratory experiments have shown that HGT occurs from plant cells to bacteria under artificial and

highly optimized conditions, but have failed to demonstrate HGT in anything resembling natural conditions (De Vries et al. 2001).

Concerns raised by critics of GM technology frequently invoke the risks stemming from HGT. It is hypothesized that DNA engineered into a plant may eventually be released from the plant upon decay, where it may be taken up by microorganisms and lead to some significant level of risk. It should be noted, however, that scientific evidence in support of such hypotheses is not forthcoming despite many years of research in this area. Conversely, the available evidence to date appears to refute these claims (Hull et al. 2000; Morel and Tepfer 2000).

Further concerns have been raised that the use of antibiotic resistance markers in GM plants may increase the advent of multi-resistance [as seen, for example, in hospitals (Levin and Andreasen 1999)] through HGT of those factors from engineered plant material to non-resistant bacteria. However, the potential magnitude of this in forest trees appears infinitesimally small, based on the fact that HGT frequencies are at best very low and selective pressure is usually not applied in GM plantations. Moreover, many such resistance factors are present in natural bacterial populations, including soil microorganisms and microorganisms that live in the human gut, even when no antibiotics are applied (Levy et al. 1988). It is illogical to assume that the effect of HGT of a resistance marker from a GM plant to a soil bacterium would be more frequent or more serious than the same transfer from a naturally occurring bacterium to that soil bacterium, in particular under circumstances where no selective advantage is conferred.

## 5.5 Development of Weedy Potential

Any change (whether it is achieved by breeding, mutation or genetic engineering) in the genetic makeup of an organism may potentially alter the behavior of this organism in its environment. It is sometimes argued that GM may introduce a trait that will provide an organism with a competitive advantage over other species, and thus plants could develop weedy potential with undesirable effects. It is instructive to consider factors mitigating against the likelihood of engineered plants becoming weeds, such as follows: (1) weeds can only develop if a selective advantage is present (Conner et al. 2003). Most improved plants aimed at industrial food and wood production are optimized for traits other than survival in the natural environment. Such organisms tend to die out when human attention is removed (Crawley et al. 2001); and (2) any new species introduction represents a new organism with tens of thousands of genes that may, collectively, lead to weedy potential. It is difficult to conceive that an established but non-invasive plant species would become a problem weed, solely due to the introduction of a single new gene.

The discussion surrounding weediness currently has a very narrow focus and lacks acknowledgement that (1) new genotypes should be assessed on the

basis of their characteristics and their impact on the environment rather than by the technology used to create them, and (2) weediness is not inherent to the GM discussion, and the risk of weediness related to conventionally bred genotypes should be considered as well and, most importantly, compared to the risks of genetically modified genotypes. Putting the issue into perspective may lead to the conclusion that the potential for weediness with regard to GM trees may not be greater, and could in fact be even lower, than currently accepted practise.

## 5.6 Pollen Flow from Transgenic Trees

The escape of transgenic pollen into wild populations of the same species, or into interfertile species, may have an effect on these populations (Rhymer and Simberloff 1996; Ellstrand 2001), with this effect being strongly dependent both on the new gene being expressed and the extent of any potential advantage being conferred upon the receiving organism. Many forest trees are wind pollinated and can fertilize each other at great distances, albeit inefficiently (Lindgren and Lindgren 1996), so the concern that they might spread transgenes to unmonitored trees is legitimate (although their potential impact is still related to the modification in question and should not be treated differently from conventional modifications). Decisions must be made on a case-by-case basis, and most importantly on scientific data rather than assumptions and fear.

Genetic modification of food crops arouses strong fears in many people and the term 'Frankenfoods' has often been used to describe GM crops. In forestry, some concerns also arise, as, for example, fungal (mycorrhizal) symbionts of trees often produce edible fruiting bodies. Also, some forest trees are important producers of honey and fruits, leading to the concern that GM material may end up in a food product. Fears that transgenes in pollen from GM trees may contaminate vegetables (particularly where organic produce is concerned) have also been expressed, as discussed in an agricultural context (Dale 1999; Quist and Chapela 2001; Conner et al. 2003). From a scientific perspective we still need to ask 'what is the risk of such gene transfer to food crops'? The simple fact that the gene may travel does not pose any risk as such, as demonstrated by the fact that millions of people have eaten and continue to eat genetically modified crops without any scientifically substantiated health risk.

Forest trees, especially those that are wind pollinated, can sometimes induce allergenic reactions in humans. For example, pollen from the Japanese Sugi tree (*Cryptomeria japonica*) causes severe allergies (Yasueda et al. 1983; Sakaguchi et al. 1990). Although it has been claimed that transgenic trees have the potential for even worse allergenic effects, scientific evidence may indicate the opposite, particularly where GM offers the potential to reduce or eliminate the allergenic effects of pollen emanating from plantations, by eliminating the production of allergenic proteins from the outer pollen wall.

## 5.7 Forest Certification

Regulatory restrictions represent another category of challenge to biotechnology applications, with the most important being the implementation of forest standards through various forest certification systems. Forest certification employs third party accreditation of timber producers to certify that timber or timber products on sale to the consumer have been produced and harvested in an environmentally responsible manner. These schemes attempt to persuade consumers to avoid purchasing products harvested from unsustainably managed forests (Nilsson 2001).

There are at least 70 certification schemes in existence, and they differ widely in their criteria and objectives. One of the biggest such certification schemes, the Forest Stewardship Council (FSC), strongly opposes genetically modified trees, even at the research stage (see Sections 6.8 and 10 at [www.fscoax.org/html/1-2.html](http://www.fscoax.org/html/1-2.html)). Not surprisingly, the FSC has come under intense scrutiny over its stance (Strauss et al. 2001). Such obstacles placed in the path of developing forest plantations are limiting vitally needed productivity gains, while effectively legitimizing the sale of lucrative exotic timber from natural forests (Freris and Laschefski 2001; Fenning and Gershenson 2002). Further, such certification schemes appear to base their decisions on political reasons rather than scientific fact, which has the potential to seriously inhibit our ability to provide environmentally friendly solutions to plantation forestry.

## 5.8 Forest Plantations: A Threat to Biodiversity?

Plantation forestry has often been criticized for a tendency to reduce biodiversity, and the application of biotechnological tools, and particularly cloning, is seen by some as potentially accelerating that trend. However, this is a matter of decision-making in forest management, and there are known forest practises that can preserve biodiversity at high levels (Burdon 2001). In this context, most plantation forests provide for higher levels of biodiversity than do agricultural plantations, which is especially pertinent where annual crops are being considered as alternative sources of fiber to trees. Also, there is growing evidence supporting the concept that the use of genetically engineered crops in agriculture can actually increase biodiversity compared to areas where conventional technology is used alone (Ammann 2004).

To conclude, many of the arguments against GM applications to plantation forest trees can be refuted on scientific and logical grounds, and/or there are suitable approaches available for mitigating and managing serious risk factors. While we must continue to debate these issues, as researchers we must also acknowledge that we have failed to convince a large proportion of the public, nor have we made substantial progress in alleviating their underlying concerns, which are often of an ethical or spiritual nature, and, consequently, not able to be addressed using scientific logic alone. Our major challenge, therefore,

is to learn to engage with the public not only as responsible scientists, but also as concerned individuals, and to be better able to discuss our science and its applications within the broader contexts of human societies and human culture. It is imperative to counter the current wave of pessimism that arises when it comes to the benefits of scientific endeavor, and to focus on the significant contributions science can make to solving environmental problems.

## 6 Conclusions

We have discussed some major challenges to conifer plantation forests through new uses of biotechnology. We are confident that most, if not all, of the technical challenges we have identified will eventually be met. With others, however, we have also identified the huge credibility gap that exists between scientists and the public, particularly with regard to GM applications, and the need for greater engagement with the critics, as well as acceptance of the important roles that ethical, environmental and social issues should play in decision-making. In addition, we have identified gaps of understanding between researchers and investors in both the attribution of value to biotechnology-based improvements and instituting the most effective mechanisms whereby these improvements may be implemented. Our discussion has emphasized the need for clarity of vision and purpose, improved communication, and the application of standard practises of good business planning and management.

## References

- Ammann K (2004) Biodiversity and agricultural biotechnology: a review of the impact of agricultural biotechnology on biodiversity. Botanical Garden, University of Bern, [http://www.croplifeasia.org/ref\\_library/biotechnology/report-biodiv-biotech3.pdf](http://www.croplifeasia.org/ref_library/biotechnology/report-biodiv-biotech3.pdf)
- An G, Watson BD, Chiang CC (1986) Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using a binary Ti vector system. *Plant Physiol* 81:301–305
- Anon (2005) Truth about trade and technology. <http://www.truthabouttrade.org/article.asp?id=3744>
- Balocchi CE (1997) Realised vs operational gain: the role of propagation strategies. In: Proc IUFRO '97 – Genetics of Radiata Pine, Forest Research Institute Bull 203, pp 253–255
- Binns AN, Thomashow MF (1988) Cell biology of *Agrobacterium* infection and transformation of plants. *Annu Rev Microbiol* 42:575–606
- Bishir J, Roberds JH (1999) On numbers of clones needed for managing risks in clonal forestry. *For Genet* 6:149–155
- Bishop-Hurley SL, Zabkiewicz RJ, Grace L, Gardner RC, Wagner A, Walter C (2001) Conifer genetic engineering: transgenic *Pinus radiata* (D. Don) and *Picea abies* (Karst) plants are resistant to the herbicide Buster. *Plant Cell Rep* 20:235–243
- Borrhalho NMG (2001) The purpose of breeding is breeding for a purpose. In: Proc IUFRO Symp on Developing the Eucalypt for the Future, Valdivia

- Brown C, Ball J (2000) World view of plantation grown wood. In: Krishnapillay B, Soepadmo E, Arshad N, Wong A, Appanah S, Chik S, Manokaran N, Tong N, Choon K (eds) Proc 21st IUFRO World Congr, vol 1, Sub-Plenary Sessions, Kuala Lumpur, pp 377–389
- Burdon RD (2001) Genetic aspects of risk – species diversification, genetic management and genetic engineering. NZ J For 44:20–25
- Burdon RD, Walter C (2004) Exotic pines and eucalypts: perspectives on risks of transgenic plantations. In: Strauss SH, Bradshaw HD (eds) The bioengineered forest: challenges for science and society. RFF (Resources for the Future) Press, Washington, DC, pp 52–75
- Burley J (2002) Tree improvement and sustainable forestry: an international perspective. In: Proc 28th Meeting of the Canadian Tree Improvement Association, Symp on Integrating Tree Improvement with Sustainable Forest Management Practices, Edmonton, Alberta, pp 1–8
- Carson MJ (1986) Advantages of clonal forestry for *Pinus radiata* – real or imagined? NZ J For Sci 16:403–415
- Carson MJ (2002) Intensive tree breeding to enhance fibre production. In: Proc 28th Meeting of the Canadian Tree Improvement Association, Symp on Integrating Tree Improvement with Sustainable Forest Management Practices, Edmonton, Alberta, pp 20–28
- Carson MJ, Vincent TG, Firth A (1992) Control-pollinated and meadow seed orchards of radiata pine. In: Proc IUFRO Conf on Mass Production of Genetically Improved Trees, Bordeaux, pp 100–109
- Casasempere A (1984) Resource development planning for less developed countries. PhD thesis, University of British Columbia, Vancouver
- Casasempere A (2004) Planning, appraisal and risk analysis in clonal forestry projects. In: Walter C, Carson MJ (eds) Plantation forest biotechnology for the 21st century. Research Signpost, Kerala, pp 363–375
- Charity JA, Holland L, Grace LJ, Walter C (2005) Consistent and stable expression of the *nptII*, *uidA* and *bar* genes in transgenic *Pinus radiata* after *Agrobacterium tumefaciens*-mediated transformation using nurse cultures. Plant Cell Rep 23:606–616
- Conner AJ, Glare TR, Nap J-P (2003) The release of genetically modified crops into the environment. Plant J 33:19–46
- Crawley MJ, Brown SL, Hails RS, Kohn DD, Rees M (2001) Transgenic crops in natural habitats. Nature 409:682–683
- Dale PD (1999) Public concerns over transgenic crops. Genome Res 9:1159–1162
- Dale PD, Clarke B, Fontes MG (2002) Potential for the environmental impact of transgenic crops. Nat Biotechnol 20:567–574
- Deak M, Kiss GB, Konez C, Dudits D (1986) Transformation of *Medicago* by *Agrobacterium* mediated gene transfer. Plant Cell Rep 5:97–100
- De la Cruz I, Davies I (2000) Horizontal gene transfer and the origin of species: lessons from bacteria. Trends Microbiol 8:128–133
- Demont M, Tollens E (2004) First impact of biotechnology in the EU: Bt maize adopted in Spain. Ann Appl Biol 145:197–207
- Devey M, Carson SD, Nolan M, Matheson C, Te Riini C, Hohepa J (2003) QTL associations for density and diameter in *Pinus radiata* and the potential for marker-aided selection. Theor Appl Genet 108:516–524
- De Vries J, Meier P, Wackernagel W (2001) The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic DNA strictly depends on homologous sequences in the recipient cells. FEMS Microbiol Lett 195:211–215
- Droege W, Puehler A, Selbitschka W (1999) Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm and field studies. Biol Fert Soils 29:221–245
- Ellstrand NC (2001) When transgenes wander, should we worry? Plant Physiol 125:1543–1545
- FAO (2005) Preliminary review of biotechnology in forestry, including genetic modification. Forest Genetic Resources Working Paper FGR/59E. FAO, Rome
- Fenning TM, Gershenzon J (2002) Where will the wood come from? Plantation forests and the role of biotechnology. Trends Biotechnol 20:291–296



- Fenning TM, Gershenzon J (2003) European agbiotech crisis? *Nat Biotechnol* 21:360
- Fillatti JJ, Sellmer J, McCown B, Haissig B, Comai L (1987) *Agrobacterium*-mediated transformation and regeneration of *Populus*. *Mol Gen Genet* 206:192–199
- Fox J (2003) Resistance to Bt toxin surprisingly absent from pests. *Nat Biotechnol* 21:258–259
- Freris N, Laschefski K (2001) Seeing the wood for the trees. *Ecologist* 31:40–43
- Fromm M, Taylor LP, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. *Nature* 319:791–793
- Gartland KMA, Kellison RC, Fenning TM (2002) Biotechnology and Europe's forests of the future. A challenge document for presentation and discussion at Forest Biotechnology Forum in Europe: Impending Barriers, Policy, and Implications, Edinburgh, pp 1–19
- Gianessi L, Sivers CS, Sankula S, Carpenter JE (2002) Plant biotechnology: Current and potential impact for improving pest management in US agriculture – An analysis of 40 case studies. National Center for Food and Agricultural Policy, Washington, USA, pp 1–76
- Gianessi L, Sankula S, Reigner N (2003) Plant Biotechnology: potential impact for improving pest management in European agriculture, a summary of nine case studies. National Center for Food and Agricultural Policy, Washington, p 15
- Grace LJ, Charity JL, Gresham B, Kay N, Walter C (2005) Insect-resistant transgenic *Pinus radiata*. *Plant Cell Reports* 24:103–111
- Griffin R, Harbard JL, Centurion C, Santini P (2000) Breeding *Eucalyptus grandis* × *globulus* and other inter-specific hybrids with high inviability – problem analysis and experience with Shell Forestry projects in Uruguay and Chile. In: Proc Symp on Hybrid Breeding and Genetics, Noosa, Queensland, pp 1–13
- Guerinot ML (2000) Plant biology enhanced: the Green Revolution strikes gold. *Science* 287:242–243
- Haines R, Walker SM (1996) Derivation of a propagation strategy. In: Dieters MJ, Matheson AC, Nikles DG, Harwood CE, Walker SM (eds) Tree improvement for sustainable tropical forestry. In: Proc QFRI-IUFRO Conf, Caloundra, Queensland, vol 2, pp 218–222
- Hargreaves C, Smith D (1994a) The effects of short-term and long-term cryopreservation on embryo maturation potential of *Pinus radiata* tissue. *Cryobiology* 31:577
- Hargreaves C, Smith D (1994b) Techniques used for cryopreservation of *Pinus radiata* embryonic tissue. *Cryobiology* 31:578
- Hossain F, Pray CE, Lu Y, Huang J, Fan C, Hu R (2004) Genetically modified cotton and farmers' health in China. *Int J Occup Environ Health* 10:296–303
- Hühn M (1992) Multiclonal mixtures and number of clones. II. Number of clones and yield stability (deterministic approach with competition). *Silv Genet* 41:205–213
- Hull R, Covey SN, Dale P (2000) Genetically modified plants and the 35S promoter: assessing the risk and enhancing the debate. *Microbiol Ecol Health Dis* 12:1–5
- Jain R, Rivera MC, Lake JA (1999) Horizontal gene transfer among genomes: the complexity hypothesis. *Proc Natl Acad Sci USA* 96:3801–3806
- James C (2006) ISAAA Brief 35: global status of commercialised biotech/GM crops: 2006. [www.isaaa.org](http://www.isaaa.org)
- Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327:70–73
- Klimaszewska K, Lachance D, Bernier-Cardou M, Ruthledge RG (2003) Transgene integration patterns and expression levels in transgenic lines of *Picea mariana*, *P. glauca* and *P. abies*. *Plant Cell Rep* 21:1080–1087
- Krattiger AF (1997) Insect resistance in crops: a case study of *Bacillus thuringiensis* (Bt) and its transfer to developing countries. International Institute for Applied Systems Analysis (IIASA), Ithaca, New York, ISAAA Briefs 2, p 42
- Kube P, Carson M (2004) A review of risk factors associated with clonal forestry of conifers. In: Walter C, Carson MJ (eds) Plantation forest biotechnology for the 21st century. Research Signpost, Kerala, pp 337–361
- Kumar S, Fladung M (2001) Gene stability in transgenic aspen (*Populus*). II. Molecular characterisation of variable expression of transgene in wild and hybrid aspen. *Planta* 213:731–740



- Leisinger KM (1996) Ethical and ecological aspects of industrial property rights in the context of GM and biotechnology. Novartis Foundation for Sustainable Development, Basel, [http://www.syngentaoundation.com/genetic\\_engineering\\_biotechnology.htm](http://www.syngentaoundation.com/genetic_engineering_biotechnology.htm)
- Levin SA, Andreasen V (1999) Disease transmission dynamics and the evolution of antibiotic resistance in hospitals and communal settings. *Proc Natl Acad Sci USA* 96:800–801
- Levy SB, Marshall B, Schluederberg S, Rouse D (1988) High frequency of antimicrobial resistance in human fecal flora. *Antimicrob Agents Chemother* 32:1801–1806
- Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene co-transformation. *Proc Natl Acad Sci USA* 100:4939–4944
- Libby WJ (1982) What is a safe number of clones per plantation? In: Heybroek H, Stephan B, von Weissenberg K (eds) Resistance to disease and pests in forest trees. In: *Proc 3rd Int Worksh on the Genetics of Host–Parasite Interactions in Forestry*, Wageningen, pp 342–360
- Libby WJ, Hood JV (1976) Juvenility in hedged radiata pine. *Acta Hort* 56:91–98
- Libby WJ, Rauter RM (1984) Advantages of clonal forestry. *For Chron* 60:145–149
- Liley B (2000) Focus on the treatment of risk in forest valuations. *NZ J For* 45:3–12
- Lindgren K, Lindgren D (1996) Germinability of Norway spruce and Scots pine pollen exposed to open air. *Silv Fenn* 30:3–9
- Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev* 58:563–602
- Matzke MA, Matzke AJM (1995) How and why do plants inactivate transgenes? *Plant Physiol* 107:679–685
- Menzies MI (1994) Propagation of radiata pine plants for plantation forestry. *Comb Proc Int Plant Prop Soc* 44:382–388
- Menzies MI, Aimers-Halliday J (1997) Propagation options for clonal forestry with *Pinus radiata*. In: Burdon RD, Moore JM (eds) Genetics of radiata pine. *NZ For Res Inst* 203:256–263
- Menzies MI, Aimers-Halliday J (2004) Propagation options for clonal forestry with conifers. In: Walter C, Carson MJ (eds) Plantation forest biotechnology for the 21st century. Research Signpost, Kerala, pp 255–274
- Meyer P (1995) Variation of transgene expression in plants. *Euphytica* 85:359–366
- Moore P (2004) Battle for biotech progress. The American Enterprise Online, [http://www.taemag.com/issues/articleid.17889/article\\_detail.asp](http://www.taemag.com/issues/articleid.17889/article_detail.asp)
- Morel J-B, Tepfer M (2000) Pour une evaluation scientifique des risques: le cas du promoteur 35S. Are there potential risks associated with use of the cauliflower mosaic virus 35S promoter in transgenic plants? *Biofutur* 201:32–35
- Nester EW, Thomashow LS, Metz M, Gordon M (2002) 100 years of *Bacillus thuringiensis*: a critical scientific assessment. *American Academy of Microbiology*, pp 1–22, <http://www.asm.org/asm/files/ccpagecontent/docfilename/0000003782/btreport%5b1%5d.pdf>
- Nielsen KM, Bones AM, Smalla K, van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? *FEMS Microbiol Rev* 22:79–103
- Nilsson S (2001) Forest policy, criteria and indicators, and certification. International Institute for Applied Systems Analysis (IIASA), Laxenburg, Rep IR-01-024, p 16
- Ochmann H, Lawrence JG, Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* 405:299–304
- Owusu RA (1999) GM technology in the forest sector, a scoping study for the WWF. [www.wwf-uk.org/filelibrary/pdf/gmsummary.pdf](http://www.wwf-uk.org/filelibrary/pdf/gmsummary.pdf)
- Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchcliffe E, Adams JL, Silverstone AR, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotechnol* 23:482–487
- Phipps RH, Park JR (2002) Environmental benefits of genetically modified crops: global and European perspectives on their ability to reduce pesticide use. *J Anim Feed Sci* 11:1–18

- Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leple JC, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Schuch W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. *Nat Biotechnol* 20:558–560
- Pray CE, Huang J, Hu R, Rozelle S (2002) Five years of Bt cotton in China: the benefits continue. *Plant J* 31:423–430
- Pua E-C, Mehra-Palta A, Nagy F, Chua N-H (1987) Transgenic plants of *Brassica napus* L. *Bio/Technology* 5:815–817
- Punja ZK (2001) GE of plants to enhance resistance to fungal pathogens – a review of progress and future prospects. *Can J Plant Pathol* 23:211–215
- Quist D, Chapela IH (2001) Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature* 414:541–543
- Rhymer JM, Simberloff D (1996) Extinction by hybridization and introgression. *Annu Rev Ecol Syst* 27:87–109
- Sakaguchi M, Inouye S, Taniai M, Ando S, Usui M, Matuhasi T (1990) Identification of the second major allergen of Japanese cedar pollen. *Allergy* 45:309–312
- Sedjo R (2004) Potential for biotechnology applications in plantation forestry. In: Walter C, Carson MJ (eds) *Plantation forest biotechnology for the 21st century*. Research Signpost, Kerala, pp 3–24
- Shahin EA, Sukhapinda K, Simpson RB, Spivey R (1986) Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA. *Theor Appl Genet* 72:770–777
- Shelbourne CJA, Carson MJ, Wilcox MD (1989) New techniques in the genetic improvement of radiata pine. *Commonwealth For Rev* 68:3
- Shin DI, Podila GK, Hunag Y, Karnosky DF (1994) Transgenic larch expressing genes for herbicide and insect resistance. *Can J For Res* 24:2059–2067
- Shintani D, DellaPenna D (1998) Elevating the vitamin E content of plants through metabolic engineering. *Science* 282:208–210
- Smith DR, Walter C, Warr A, Hargreaves CL, Grace LJ (1994) Somatic embryogenesis joins the plantation forestry revolution in New Zealand. In: *Proc Tappi Biol Sci Symp*, Minneapolis
- Snow A (2002) Transgenic crops – why gene flow matters. *Nat Biotechnol* 20:542
- South DB (1999) How can we feign sustainability with an increasing population? *New For* 17:193–212
- Spangenberg G, Neuhaus G, Schweiger HG (1986) Expression of foreign genes in a higher plant cell after electrofusion-mediated cell reconstitution of a microinjected karyoplast and a cytoplasm. *Euro J Cell Biol* 42:236–238
- Stewart CN, Richards HA, Halfhill MD (2000) Transgenic plants and biosafety: science, misconceptions and public perception. *BioTechniques* 29:832–843
- Strauss SH, Coventry P, Campbell MM, Pryor SN, Burley J (2001) Certification of genetically modified forest plantations. *Int For Rev* 3:87–104
- Sutton BCS, Attree SM, El-Kassaby YA, Grossnickle SC, Polonenko DR (2004) Commercialisation of somatic embryogenesis for plantation forestry. In: Walter C, Carson MJ (eds) *Plantation forest biotechnology for the 21st century*. Research Signpost, Kerala, pp 275–301
- Sweet GB (1992) Seed orchard research and management in the 1990s – a New Zealand Case Study. In: *Proc IUFRO Conf on Mass Production of Genetically Improved Trees*, Bordeaux, pp 110–118
- Tang W, Newton RJ (2003) Genetic transformation of conifers and its application in forest biotechnology. *Plant Cell Rep* 22:1–15
- Tang W, Tian Y (2003) Transgenic loblolly pine (*Pinus taeda* L.) plants expressing a modified delta-endotoxin gene from *Bacillus thuringiensis* with enhanced resistance to *Dendrolimus punctatus* Walker and *Cryptorhiza formosicola* Staud. *J Exp Bot* 54:835–844
- Taverne D (2005) *The march of unreason. Science, democracy, and the new fundamentalism*. Oxford University Press, Oxford

- Tepfer D, Garcia-Gonzales R, Mansouri H, Seruga M, Message B, Leach F, Mirna Curkovic P (2003) Homology-dependent DNA transfer from plants to a soil bacterium under laboratory conditions: implications in evolution and horizontal gene transfer. *Transgenic Res* 12:425–437
- Thompson Campbell FT (2000) Genetically engineered trees: questions without answers. American Lands Alliance, Washington, DC, [www.americanland.org/forestweb/gettrees.htm](http://www.americanland.org/forestweb/gettrees.htm)
- Van den Belt H (2003) Debating the precautionary principle: “guilty until proven innocent” or “innocent until proven guilty”? *Plant Physiol* 132:1122–1126
- Vasil IK (2003) The science and politics of plant biotechnology – a personal perspective. *Nat Biotechnol* 21:849–851
- Walter C (2004) Genetic engineering in conifer forestry: technical and social considerations. In *Vitro Cell Dev Biol-Plant* 40:434–441
- Walter C, Fenning T (2004) Deployment of genetically-engineered trees in plantation forestry – an issue of concern? The science and politics of genetically modified tree plantations. In: Walter C, Carson MJ (eds) *Plantation forest biotechnology for the 21st century*. Research Signpost, Kerala, pp 423–446
- Walter C, Grace LJ, Wagner A, Walden AR, White DWR, Donaldson SS, Hinton HH, Gardner RC, Smith DR (1998) Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. *Plant Cell Rep* 17:460–468
- Walter C, Grace LJ, Donaldson SS, Moody J, Gemmell JE, van der Maas S, Kwaalen H, Loenneborg A (1999) An efficient biolistic transformation protocol for *Picea abies* (L) Karst embryogenic tissue and regeneration of transgenic plants. *Can J For Res* 29:1539–1546
- White TL, Carson MJ (2004) Breeding programs of conifers. In: Walter C, Carson M J (eds) *Plantation forest biotechnology for the 21st century*. Research Signpost, Kerala, pp 61–85
- Wilcox PL, Richardson TE, Carson SD (1997) Nature of quantitative trait variation in *Pinus radiata*: insights from QTL detection experiments. In: Burdon RD, Moore JM (eds) *Proc IUFRO '97: Genetics of Radiata Pine*, Rotorua, NZ FRI 203, pp 304–312
- Wilcox PL, Carson SD, Richardson TE, Ball RD, Horgan GP, Carter P (2001) Cost-benefit analysis of marker based selection in seed orchard production populations of *Pinus radiata*. *Can J For Sci* 31:2213–2224
- Xhiao-hua S, Bing-yu Z, Quin-Jun H, Lie-jian H, Xiang-hua Z (2003) Advances in tree genetic engineering in China. In: *Proc 12th World Forestry Congr*, Quebec City, <http://www.fao.org/docrep/article/wfc/xii/0280-b2.htm>
- Yasueda H, Yui Y, Shimizu T, Shida T (1983) Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J Allergy Clin Immunol* 71:77–86
- Zhu S, Tomberlin D, Buongiorno J (1999) Global forest products consumption, production, trade and prices: global forest products model projections to 2010. *FAO Global Forest Product Outlook Study Working Pap GFPOS/WP/01*
- Zobel B (1993) Clonal forestry in the eucalypts. In: Ahuja MR, Libby WJ (eds) *Clonal forestry 2. Conservation and application*. Springer, Berlin Heidelberg New York, pp 139–148
- Zobel BJ, Talbert J (1984) *Applied forest tree improvement*. John Wiley, New York

**Section III Beverage Crops**

## III.1 Coffee

N. SANTANA<sup>1</sup>, R. ROJAS-HERRERA<sup>2</sup>, R.M. GALAZ-ÁVALOS<sup>1</sup>, J.R. KU-CAUICH<sup>1</sup>, J. MIJANGOS<sup>1</sup>, and V.M. LOYOLA-VARGAS<sup>1</sup>

### 1 Introduction

Modern agriculture may face tremendous challenges in the 21st century to produce sufficient food to keep pace with the growing world population. Limitations to food production include diminishing arable land and water, compounded by deforestation and an increase in the number of chemicals banned from agricultural practice. To increase crop productivity, plant scientists have devoted much effort to searching for new varieties resistant to pests and/or adverse environments.

In coffee, the seed-to-seed cycle usually takes about 4 years. To generate a desirable coffee genotype with stable, improved traits using conventional breeding requires at least six self-pollination cycles. More than 30 years may be needed to generate an improved coffee variety (Carneiro 1999). This time-consuming constraint has prompted the search for alternative approaches to the genetic improvement of coffee.

### 2 Somatic Embryogenesis in Coffee

Somatic embryogenesis (SE), like many biological processes, is affected by numerous factors, the most important of which are explant origin, physiological status of the donor plant, growth regulators used for induction, components of the culture media (especially nitrogen source) and pH, and incubation conditions.

#### 2.1 Growth Regulators in SE

The effect of different growth regulators on the process of SE depends on a strong interrelationship between the composition and strength of the culture

---

<sup>1</sup>Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, México, e-mail: vmloyola@cicy.mx

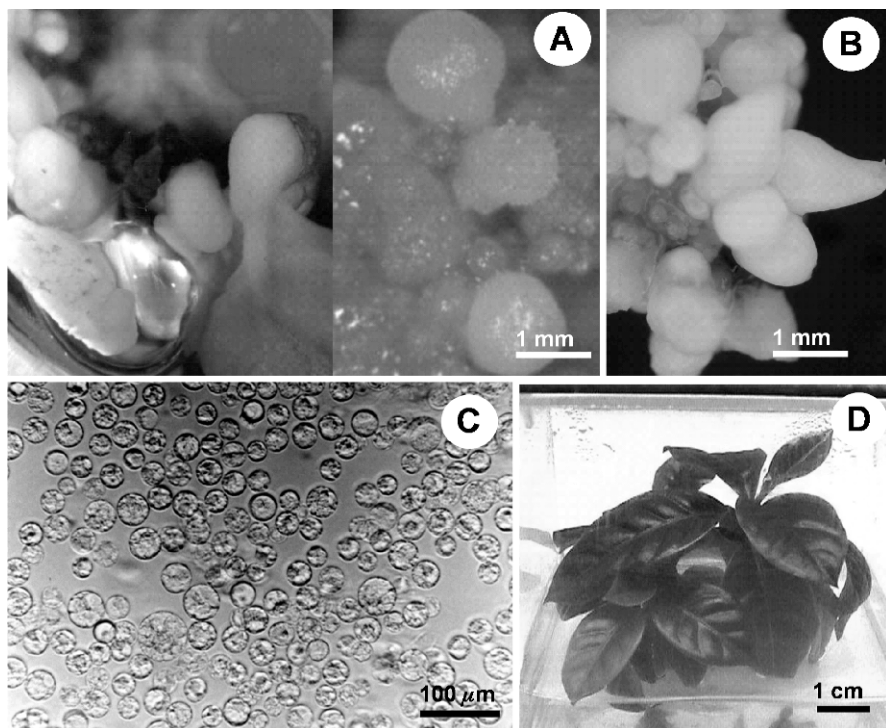
<sup>2</sup>Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, Av. Normalistas 800, Guadalajara, Jalisco, México

medium used, the amount and type of the carbon source, and the environmental conditions employed during the incubation of the tissues. Various cultured tissues from different *Coffea* species possess a high embryogenic potential. SE has been induced from different explants, including ortotropic and plagiotropic shoots (Staritsky 1970; Nassuth et al. 1980; Raghuramulu et al. 1987), foliar tissue (Söndahl and Sharp 1977; Dublin 1981; Quiroz-Figueroa et al. 2001, 2002; Cid et al. 2004), the outer covering of ovules (tegument) (Lanaud 1981), somatic tissue of anthers (Ascanio and Arcía 1987) and from perisperm (Sreenath et al. 1995). Nevertheless, leaves are so far the most widely used explant owing to their abundance, year-round accessibility and continuous production.

Pioneer work on SE in coffee was conducted by Staritsky (1970), who cultured internodal segments of ortotropic and plagiotropic shoots from *Coffea arabica* L., *C. canephora* Pierre ex Froehner (Robusta) and *C. liberica* Bull ex Hiern. Explants were cultured on media described by Heller (1953) and Linsmaier and Skoog (LS) (1965), supplemented with different growth regulators, including kinetin (0.46  $\mu\text{M}$ ), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.45  $\mu\text{M}$ ) or  $\alpha$ -naphthaleneacetic acid (NAA, 5.37  $\mu\text{M}$ ). In 1975, Herman and Haas (1975) reported the formation of organoids from leaf-derived calli grown on modified LS medium supplemented with 0.46  $\mu\text{M}$  kinetin and 0.45  $\mu\text{M}$  2,4-D. These structures developed roots in Gresshoff and Doy (GD) medium (Gresshoff and Doy 1972) containing 0.53  $\mu\text{M}$  NAA (Herman and Haas 1975).

A protocol to obtain calli with high embryogenic potential from leaf explants of *C. arabica* cv. Bourbon was reported by Söndahl and Sharp (1977) and Söndahl et al. (1979). In this protocol, two different media for 'conditioning' and 'induction' were employed. The success in the induction of high frequency SE (HFSE) was shown to be affected by the concentration of growth regulators in the conditioning medium.

In most systems involving the induction of SE described so far, the concentration of auxin is a limiting factor for embryogenic callus induction (Halperin and Wetherell 1965; Söndahl and Sharp 1977). In coffee, the presence of 2,4-D in combination with kinetin markedly increased HFSE (>60%) in the conditioning medium. Other auxins, such as indole-3-butyric acid (IBA) and NAA combined with kinetin, were not effective for HFSE induction (10–20%), but they increased low frequency SE (LFSE) induction up to 60%, especially when NAA was used. Depletion of 2,4-D and reducing the concentration of NAA and kinetin in the induction medium appeared to be essential for somatic embryo development in *Coffea* spp. The regulatory mechanism of the preferential and promoting role of 2,4-D in the onset and sequential development of HFSE is not clear. Although calli induced by NAA and 2,4-D can be phenotypically similar, the physiological and biochemical status of two types of callus may be different. More research is required to elucidate the biochemical, molecular, cytological and physiological aspects of SE in coffee during its induction in relation to auxin (Söndahl et al. 1985).



**Fig. 1.** Developmental pathway for SE in coffee. **A** *Left panel:* direct SE. *Right panel:* indirect SE. **B** Somatic embryos produced by leaf calli from *C. arabica*. **C** Protoplast obtained from cell suspension cultures of *C. arabica*. **D** *C. arabica* plant clonally propagated (photo from author's laboratory)

Two developmental patterns are clearly discernible in coffee SE (Fig. 1A). One is direct SE, where embryos are directly formed from the explant (Quiroz-Figueroa et al. 2002), and is associated from the beginning with determined proembryogenic cells (DPEC). The other pattern is indirect SE, where embryos are derived from an embryogenic dedifferentiated tissue (callus), and is associated with dedifferentiated cells that require redifferentiation, proliferation and the induction of determined embryogenic cells (Söndahl et al. 1985).

A marked effect of indole-3-acetic acid (IAA) during SE induction was observed by Dublin (1980b) using explants of Arabusta (F1 hybrid from *C. arabica* × *C. canephora*, 4X). The presence of 2.85 μM IAA and 0.46 μM kinetin was shown to be favourable for the development of somatic embryos. Direct SE was also observed in a cytokinin-rich medium in the absence of auxins, whereas 2,4-D was required during callus induction for obtaining indirect SE. Suppressing 2,4-D and adding benzyladenine (BA) resulted in the formation of embryos (Dublin 1981). Similar results have been reported by Owuor (1987). Yasuda et al. (1985) reported a simple method of SE induction in leaf explants from *C.*



*arabica* var. Typica using 5  $\mu\text{M}$  BA as the sole growth regulator. The regenerative capacity of calli could be maintained for more than 2 years. In *C. arabica* var. Caturra and five Catimor lines, the medium containing 4.52–13.56  $\mu\text{M}$  2,4-D and 4.43–44.38  $\mu\text{M}$  BA promoted formation of embryogenic calli (Santana et al. 1988). For embryo development, cultures were transferred first to medium containing 2.32  $\mu\text{M}$  kinetin and 0.53– $\mu\text{M}$  NAA, followed by medium with 0.46  $\mu\text{M}$  kinetin and 2.46– $\mu\text{M}$  IBA (Fig. 1B).

De García and Menéndez (1987) explored the hormonal requirements for SE induction in *C. arabica* cv. Catimor using different combinations of cytokinin [kinetin, BA or 2-isopentenyladenine (2iP)] and auxin (2,4-D or IBA). Proembryogenic calli formed on explants grown on medium containing 35.5  $\mu\text{M}$  BA and 4.52  $\mu\text{M}$  2,4-D, but the formation of somatic embryos (75 embryos/explant) was observed after cultures were transferred to a medium supplemented with 10% coconut milk. HFSE was observed when the medium was supplemented with 10  $\text{mg l}^{-1}$  of hydrolyzed casein, 26.6  $\mu\text{M}$  IBA and 4.92  $\mu\text{M}$  2iP. The conversion of embryos to plantlets was achieved in medium containing 10% coconut milk and 0.16  $\mu\text{M}$  NAA under constant light (60  $\text{cd m}^{-2}$ ) at 28 °C.

In a study dealing with the effect of factors affecting SE in coffee, Marques (1987) observed increased callus growth by 60–70% after reducing the concentrations of nitrogen and increasing the concentrations of boron and magnesium. According to the author, embryo proliferation was favored by a higher osmolarity of the culture medium owing to the presence of mannitol and casein hydrolysate, and incubation of cultures in the dark. We also observed similar results after decreasing the nitrogen concentration in the medium (Fuentes-Cerda et al. 2001). The use of amino acids and reduced organic nitrogenous compounds also promoted SE of coffee (Fuentes-Cerda et al. 2001). Asparagine was shown to be effective in SE induction (Nishibata et al. 1995). SE in coffee could also be promoted by exogenous application of other nitrogenous compounds such as polyamines (Calheiros et al. 1994).

A comparative study of the culture response of the embryogenic (KF 2.1, KF 2.6 and ET 25) and non-embryogenic (ET 20.1) genotypes of *C. arabica* was conducted to investigate why SE did not occur in non-embryogenic (non-reactive) genotypes (Michaux-Ferrière et al. 1989). It was found that two clusters of embryogenic cells were formed on explants of embryogenic genotypes that successively appeared on the callus. The first clusters formed at the edge of the callus after 20 days of culture, but the embryogenic characteristics were lost thereafter. The second clusters usually appeared after 60 days of culture. They comprised mainly embryogenic cells that were formed through dedifferentiation and eventually extended to the whole callus. Isolated cells from these clusters developed into polarized proembryos and later bipolar structures (somatic embryos). In non-embryogenic ET 20.1 genotype, embryogenic cells were also detected after 60 days of culture. However, these cells were not capable of producing somatic embryos, although the calli possessed similar cytological characteristics as those of embryogenic callus.

Hatanaka et al. (1991) reported that all auxins tested (NAA, IBA, IAA and 2,4-D) were inhibitory to somatic embryo formation of Robusta but were promoted by cytokinin such as 2iP, BA and kinetin at 5  $\mu\text{M}$ . Under these conditions, over 100 embryos per explant were formed. Using the modified version of the method described by Söndahl and Sharp (1997), Neuenschwander and Baumann (1992) reported efficient embryo formation from calli of *C. arabica* grown on conditioning medium with 2,4-D (4.5  $\mu\text{M}$ ) and kinetin (18.4  $\mu\text{M}$ ). After cultures were transferred to a liquid induction medium of reduced ionic strength with 0.23  $\mu\text{M}$  NAA and 2.7  $\mu\text{M}$  kinetin for 18–24 weeks, somatic embryos were formed in a highly synchronized fashion. These embryos possessed a high germination rate of 94.5% and were designated self-controlled SE (SCSE). The protocol may be important for coffee propagation using bioreactors.

The friable embryogenic tissue (FET) from coffee leaf explants was also a good source of somatic embryos (Söndahl and Noriega 1992). Culture grew well in the medium supplemented with 1.13  $\mu\text{M}$  2,4-D and 0.45  $\mu\text{M}$  kinetin, where the first globular embryos were observed after 4 weeks. Growth in culture increased 20-fold after transfer to new medium containing 9.83  $\mu\text{M}$  abscisic acid (ABA) for 12 weeks. Results of the preliminary study indicated that 0.2 g fresh weight of FET could give rise to as many as 2500 embryos. Aponte (1993) also generated pro-embryogenic tissues (PET) from different coffee genotypes using two different media. One was LIQC-3 medium comprising half-strength MS (Murashige and Skoog 1962) basal salts, organic constituents from B5 medium, 30  $\text{g l}^{-1}$  sucrose and 13.31  $\mu\text{M}$  BA, while the other was NAR1/4 medium that contained one-quarter-strength MS basal salts, organic constituents from B5 medium (Gamborg et al. 1968), 30  $\text{g l}^{-1}$  of sucrose and 4.92  $\mu\text{M}$  2iP. From nine genotypes tested, five generated PET at high frequency. For F5.302 genotype, it was observed that NAR1/4 was more effective than LIQC-3 for PET formation after 2 months of culture. In addition, 2iP was preferable to BA for the induction of SE in the phenotypes tested. Similar results were also reported previously (Hatanaka et al. 1991). In addition, the gelling agent in the medium could influence the embryogenic response of *C. arabica* (Bieysse et al. 1993).

Van Boxtel and Berthouly (1996) reported an efficient protocol for the induction, proliferation and regeneration of embryogenic calli from leaf explants of *C. arabica*, *C. canephora* and the hybrids Arabusta (*C. arabica*  $\times$  *C. canephora*) and Congusta (*C. congensis*  $\times$  *C. canephora*). Cultured cells proliferated rapidly in a liquid medium containing MS salts and 4.5  $\mu\text{M}$  2,4-D, with an inoculum density of 10  $\text{g l}^{-1}$  and subculture every 7 days. The embryogenic potential could be retained for about 2 years by culturing the cell clusters 250–1,000  $\mu\text{m}$  in size at a density of 5  $\text{g l}^{-1}$  with transfer every 3–4 weeks. Depletion of 2,4-D and reducing the inoculum density to 1  $\text{g l}^{-1}$  resulted in the proliferation of embryogenic calli. Using this procedure, 1 g of callus from *C. canephora* or Arabusta yielded  $1.2 \times 10^5$  or  $0.9 \times 10^5$  somatic embryos, respectively, after 8–10 weeks of culture. It has shortened the process from culture initiation to em-

bryo regeneration by 4–6 months. In contrast, *C. arabica* genotypes were least responsive to the procedure. In another study, leaf explants of *C. canaphora* were induced to form highly embryogenic callus on medium containing 2,4-D, IBA and 2iP (Berthouly and Michaux-Ferrière 1996). Subsequently, the callus gave rise to 80,000 embryos with a high capacity for conversion to plants.

For induction of direct embryogenesis, we have shown that preconditioning the source plants by culture on medium with 0.54  $\mu\text{M}$  NAA and 2.32  $\mu\text{M}$  kinetin for 2 months is critical for an embryogenic response (Quiroz-Figueroa et al. 2002). There was no embryogenic response when explants were excised from the source plants grown in the absence of growth regulators. Using the leaf explants, the presence of triacontanol in half-strength MS basal medium containing 1.1  $\mu\text{M}$  BA and 2.28  $\mu\text{M}$  IAA induced direct SE, which gave rise to a maximum of 260 somatic embryos per culture for *C. arabica* and 59 for *C. canephora* (Giridhar et al. 2004a).

The embryogenic response also varied with the position of the leaves, with a poor response from the first two pairs of leaves from the apex. In a given leaf, explants that originated from the distal end were less responsive than those from the basal end. Santana (1993) also reported the influence of leaf position and age on embryogenic callus formation in *C. canaphora* var. Robusta and *C. arabica* vars. Caturra and Catimor 9722. Leaf explants from nodes 2, 3 and/or 4 of all genotypes tested were most effective in the formation of embryogenic callus. In addition, ortotropic branches generally were more responsive than plagiotropic branches. In *C. canaphora* var. Robusta, the age and cellular structure of calli, inoculum density and medium composition were important factors affecting somatic embryo formation in suspension cultures (Montes et al. 1995). The use of 2- to 3-month-old callus in conjunction with an inoculum density of 2–3  $\text{g l}^{-1}$  was optimal for establishing embryogenic suspensions.

The embryogenic capacity of coffee is genotype dependent, and this character is fixed in early, F3 or F4 generations (Molina et al. 2002). In view of this, the embryogenic capacity for F5 lines can be predicted in a breeding program if the F3 or F4 ancestors, or lines with the same parents on those generations, are evaluated. The embryogenic capacity amongst genotypes varied greatly, ranging from 4.8 to 72.7% (Molina et al. 2002). There is a relationship between the embryogenic response of the progenies and their progenitor. Furthermore, significant differences are also detected in embryogenic responses depending on the physiological stage of the source plant from which leaf explants are collected, particularly when the plant changes from the vegetative to the reproductive stage (Santana-Buzzy et al. 2004).

Hatanaka et al. (1995) investigated the effect of ethylene on SE in *C. canaphora*. The presence of inhibitors of either ethylene production or ethylene action affected embryo formation, indicating the possible regulatory role of ethylene in SE of this species. These findings are supported by the results of other studies showing that the presence of 30–60  $\mu\text{M}$   $\text{AgNO}_3$  increased the production of somatic embryos in five genotypes of *C. canephora* (Fuentes et al. 2000). How-

ever, higher  $\text{AgNO}_3$  concentrations were inhibitory. In addition to ethylene inhibitors, SE is affected by the carbon source in the medium. Thus, substitution of sucrose by fructose resulted in a significant increase in somatic embryos in the coffee genotypes N91 and N128, while maltose was highly effective for the genotype N75. In N91, more synchronous embryo development was observed when glucose replaced sucrose (Fuentes et al. 2000). Hypocotyl explants of both *C. arabica* and *C. canephora* were used to produce direct somatic embryos in the presence of  $\text{AgNO}_3$  (Giridhar et al. 2004b). These authors reported the production of a maximum of 144 and 69 embryos per explant of *C. canephora* and *C. arabica*, respectively, in MS medium with 1.1  $\mu\text{M}$  BA, 2.85  $\mu\text{M}$  IAA and 40  $\mu\text{M}$   $\text{AgNO}_3$  (Giridhar et al. 2004b).

The growth of embryogenic cell suspension is also affected by salicylic acid (SA), although the effect is concentration-dependent. In *C. arabica* cv. Caturra Rojo, the presence of SA at  $10^{-12}$  and  $10^{-10}$  M increased cellular growth and SE by two-fold (Quiroz-Figueroa et al. 2001). The increase in the number of somatic embryos could be a reflection of an increase in the number of embryogenic cells induced by SA. In addition to SA, dissolved oxygen (DO) has been shown to affect somatic embryo development of *C. arabica* cv. Catimor 9722. The number of somatic embryos was more in the presence of 80% DO, but the majority of embryos were globular- and heart-shaped and only 6.6% was torpedo-shaped (De Feria et al. 2003). However, the number of torpedo-shaped embryos increased up to 20% when the DO concentration was decreased by 50%. These results suggested that higher DO concentrations favoured the differentiation of globular- and heart-shaped embryos, while torpedo-shaped embryos developed at lower DO concentrations.

Zamarripa et al. (1991b) developed a protocol for mass propagation of coffee in liquid medium. Embryo formation was affected by the inoculum density. SE could be inhibited when the inoculum density was high. However, the inhibition could be partially suppressed by periodically renewing the medium. The cause of this phenomenon is not clear, but, based on the culture response of *Citrus* (Gavish et al. 1992), it is assumed that inhibition may be associated with the presence of SE proteins in the media that inhibit SE. Nevertheless, growing embryogenic tissues of *C. canephora* in Erlenmeyer flasks or a bioreactor could yield up to 200,000 somatic embryos (Zamarripa et al. 1991a, b). Ducos et al. (1993) reported the production of 600,000 embryos per liter of medium in a bioreactor. However, unlike embryogenic tissues, explants of *C. arabica* grown under the same culture conditions produced fewer embryos.

A micropropagation method known as an automated temporary immersion system (RITA: recipient à immersion temporaire automatique) has been shown to be beneficial for the production and germination of somatic embryos of several plant species, including coffee (Etienne and Berthouly 2002). Berthouly et al. (1995) and Etienne et al. (1997) reported a protocol for virtually synchronous production and germination of somatic embryos of *C. arabica* F<sub>1</sub> hybrids using RITA, without the need for selection before acclimatization. Depending on the genotypes, the yield of somatic embryos ranged

from 15,000 to 50,000 per gram of embryogenic suspension cell mass. The population of normal torpedo-stage embryos produced using RITA is usually >90%. Moreover, hyperhydricity can be overcome using short immersion times (1 min) (Etienne and Berthouly 2002). Albarrán et al. (2005) showed that the quality of the embryos produced in the RITA devices depends on the frequency and time of immersions. The use of 1-min immersions every 4 h could lead to the production of the maximum number of torpedo-shaped embryos without hyperhydricity and with high frequency (75%) of conversion into plants.

Apart from somatic embryo production and germination, somatic embryos produced by RITA can be sown directly in soil. Using germinated embryos, direct sowing resulted in a high frequency conversion of embryos into plants. A culture density in excess of 1600 embryos in a bioreactor positively affected embryo morphology by promoting embryonic axis elongation. At this density, the addition of a high concentration of sucrose (234 mM) 2 weeks before sowing promoted plant conversion in soil (78%) and vigorous vegetative growth of the plants. Furthermore, direct sowing reduced handling time to 13% and shelving area requirements to 6.3% of the values obtained by conventional acclimatization of plants on gelled media (Etienne-Barry et al. 1999).

The clonal fidelity of regenerated trees has been assessed in large-scale field trials in order to confirm the propagation technology for *C. canephora* Pierre var. Robusta via SE in liquid medium. A total of 5067 trees originating from 5- to 7-month-old embryogenic cell suspension cultures were planted in the Philippines and Thailand and compared with trees derived from in vitro axillary budding (microcuttings). In morphological traits and yield characteristics, there were no significant differences between the plants derived from embryogenic tissues and microcuttings. After three harvests in Thailand, the most productive lines showed a cumulative yield of green coffee of more than 3,000 kg ha<sup>-1</sup>. However, somaclonal variation at the DNA level or the occurrence of some phenotypic variants that could appear later cannot be excluded (Ducos et al. 2003). Nevertheless, high frequency embryo formation in explants from adult coffee plants renders this species an attractive system for perennial tropical crops of economic importance.

### 3 Other Tissue Culture Methods

#### 3.1 Protoplast Culture

To date, coffee cultivation is restricted to only a few cultivars, rendering a narrow genetic base to the crop. For coffee improvement, it is imperative to broaden the genetic variability of the crop. Conventional breeding to generate new coffee genotypes with increased yield, improved grain quality and increased resistance to pests and diseases is time-consuming and laborious. The process can

take in excess of 30 years to deliver a new variety (Carneiro 1997). In addition, coffee breeding is complicated by genetic barriers that exist in some genotypes, thereby decreasing the efficiency of interspecific or intergeneric hybridization. According to Mazzafera and Carvalho (1992), the difficulty in crossing species belonging to the section *Paracoffea* and *Mascarocoffea* with those from the section *Eucoffea* is the limiting factor for obtaining genotypes with increased resistance to biotic or abiotic extremes, or with a low caffeine content.

To overcome the difficulties encountered by breeding, biotechnological approaches offer an alternative to produce interspecific or intergeneric hybrids through protoplast fusion, or novel plants by genetic transformation, within a relatively short period of time (Söndahl et al. 1981). In coffee, protoplast fusion permits the introduction of desirable characteristics from *C. canephora* or other diploid species to *C. arabica* (Nyange et al. 1997a). This has potential for coffee improvement by the production of interspecific hybrids with novel traits, such as resistance to coffee rust (Fig. 1C).

Coffee protoplasts can be isolated from various tissues, including leaves (Orozco and Schieder 1982, 1984), leaf-derived calli (Söndahl et al. 1980), somatic embryos (Schöpke et al. 1987), cell suspensions from embryogenic leaf-derived calli (Acuna and de Pena 1991; Spiral and Petiard 1991), hypocotyl-derived calli (Nyange et al. 1997a) and non-embryogenic root-derived calli (Grèzes et al. 1994). Yasuda et al. (1987) reported the isolation of protoplasts from embryogenic calli of *C. arabica* using 0.2% Pectolyase Y23, 1% Cellulase Onozuka RS and 0.5 M mannitol. Protoplasts cultured on a semi-solidified medium containing 5  $\mu$ M BA (or 2iP) and 0.5 M mannitol underwent first cell divisions after 3 days of culture and formed colonies after 4 weeks. Depletion of mannitol accelerated colony growth and the formation of embryos, which subsequently developed into plantlets after transfer to cytokinin-containing medium. Plants were also regenerated from protoplasts isolated from somatic embryo-derived suspension cultures of *C. canephora* (Schöpke et al. 1987) and *C. arabica* cv. Caturra (Acuna and de Pena 1991) and embryogenic suspension cultures of *C. canephora*, *C. arabica* and the interspecific sexual hybrid Arabusta (Spiral and Petiard 1991).

### 3.2 Factors Affecting Protoplast Culture

Several factors are known to affect the recovery of viable protoplasts and their regeneration into plants. These include culture conditions, physiological status of the source of explants and type and concentration of cell wall degrading enzymes. Toruan-Mathius (1992) reported that a high protoplast yield could be achieved using leaves of young *C. arabica* plants grown under glasshouse conditions. In contrast, the protoplast yield was low using leaf tissue from in vitro-grown plants. Nevertheless, protoplasts cultured in liquid medium (Kao and Michayluk 1975) containing 4.43  $\mu$ M BA and 0.53  $\mu$ M NAA formed callus which, subsequently, gave rise to somatic embryos after transfer to MS medium



containing 8.86  $\mu\text{M}$  BA and 80.57  $\mu\text{M}$  IAA. However, plants that developed from somatic embryos were morphologically abnormal.

Grèzes et al. (1994) also reported low yields of viable *C. arabica* protoplasts, but yields were increased using a cell wall degrading enzyme mixture comprising 1% cellulase R10, 0.5% Driselase and 1.8% Macerozyme R10. Protoplast yield was also affected by the physiological status and cell age of source material. When 5- to 8-day-old cell suspensions were used, 75–95% of cells became protoplasts (Grèzes et al. 1994). Viable protoplasts of  $3.5\text{--}4.6 \times 10^6$  per gram (fresh weight) of cultured cells could be achieved, representing a 10- to 15-fold increase compared to that reported by Acuna and de Pena (1991). However, cell suspensions less than 5 days old were not suitable material for protoplast culture. Tahara et al. (1994) showed that the presence of 0.5 M mannitol facilitated the isolation of protoplasts from embryogenic calli of *C. arabica*. Cultures grown in MS medium supplemented with 5  $\mu\text{M}$  BA gave rise to embryos, which were capable of developing into plants.

Protoplasts are a useful tool for studying the mechanism of action of fungal toxins at the cellular level. They have been used in bioassays of crude or partially purified extracts of fungal toxins to compare the different responses between sensitive and resistant genotypes. The feasibility of using the approach to produce disease-resistant plants has been demonstrated previously (Mattern et al. 1978; Shepard et al. 1980; Wenzel and Foroughi-Wehr 1990; Möllers et al. 1992). Using this method, Nyange et al. (1997b) reported the production of coffee plants resistant to *Collectotrichum kawaiae*, the causal agent of coffee berry disease (CBD). In this study, the response was evaluated of cells and protoplasts from sensitive and resistant *C. arabica* genotypes to partially purified culture filtrates (PPCFs) of *C. kawaiae*. Results showed a differential response of the resistant and susceptible protoplasts to PPCFs, suggesting that PPCFs could be used for screening coffee genotypes resistant or tolerant to CBD (Nyange et al. 1997b).

### 3.3 Anther/Microspore Culture

Anthers (Sharp et al. 1973; Ascanio and Arcía 1987; Raghuramulu 1989) and isolated microspores (Carneiro 1997) can be cultured to produce haploid plants, which, in turn, can be used to produce homozygous plants (dihaploids) within one generation by doubling the chromosome number of the haploids. Homozygous plants are important for breeding programmes. Furthermore, masses of haploid and embryogenic single cells are perfect targets for genetic transformation (Carneiro 1997; Neuhaus et al. 1998) and for in vitro selection. In coffee, interest in the use of haploids in genetic improvement is largely due to their value for interspecific hybridization. The production of dihaploids from a heterozygous diploid species of *Coffea* allows hybridization with *C. arabica* (Ascanio and Arcía 1987) to produce homogeneous hybrids.



The first attempt to produce haploid coffee plants was reported in *C. arabica* (Sharp et al. 1973). Cultured anthers produced both haploid and dihaploid tissues and proembryo formation was also observed. However, further growth and development of proembryos was arrested. Sharp et al. also reported callus formation from anthers of the vars. Mundo Nuovo and Bourbon Amarelo, and there was a correlation between the different developmental stages of anthers, size of flower buds and quantity of calli. Monaco et al. (1977) cultured anthers of different coffee species, but only *C. liberica* was responsive, giving rise to friable callus. Neuenschwander et al. (1993) reported the regeneration of haploid cell colonies from microspores of *C. arabica*, which is the first step in the establishment of an efficient haploid plant regeneration system. Androgenesis has also been induced in *C. arabica* var. Catuai and the progeny of var. Catimor by culturing isolated microspores and anthers in either liquid or semi-solid medium (Carneiro 1997). The regeneration of haploid plants is not restricted to male gametes. Lanaud (1981) reported SE using ovules from *C. canephora* and established the culture conditions for rapid multiplication and differentiation of embryogenic masses.

When the genetic pool of *C. arabica*, which is a tetraploid species, is reduced by half, its amphiploid character guarantees the plants behave as true haploids, yet are dihaploids. Ascanio and Arcía (1994) studied the effect of the developmental stage of anthers from *C. arabica* var. Guernica and heat shock treatment on embryogenic callus formation. Floral buds of 3–4 mm in size with uninucleated, mitotic and binucleated pollen grains were treated at different temperatures (5 and 25 °C for 24 and 48 h). Callus formation was observed only on anthers with uninucleate pollen grains that possessed the dihaploid complement of chromosomes. However, treatment of anthers at 5 °C for 24 or 48 h promoted embryogenesis. Embryos that germinated and developed their first pair of leaves were grafted onto newly germinated plantlets of *C. canephora* and the chromosome number was duplicated with colchicine. Tetraploid plants were grown directly in soil for further evaluation.

## 4 Micropropagation

In general, there are two methods of coffee micropropagation, through morphogenesis and microcuttings (Dublin 1980a). The advantages and disadvantages of each methodology, as well as the phenomenon on plagiotropic reversion that occurs during micropropagation, were discussed previously (Dublin 1984). The first attempt at clonal propagation of *C. arabica* by breaking the latency of axillary buds was reported by Custers (1980). In this study, several important factors were studied, including plant age and nodal position. Plants at 2 months old are more suitable for micropropagation than younger plants. It was observed that explants originated from the 7th node to the apical meristem decreased shoot production by up to 50%. However, a multiplication rate of 13

could be obtained from explants grown on LS medium (Linsmaier and Skoog 1965) containing 44.38  $\mu\text{M}$  BA every 6 months.

For clonal propagation of *C. arabica*, a multiplication rate of three could be obtained by culture on MS medium containing 6.64  $\mu\text{M}$  BA in the first step and 13.29  $\mu\text{M}$  BA and 0.5  $\text{mg l}^{-1}$  giberelic acid in the second step (Chatterjee et al. 1991). De García and Rafael (1989) employed high concentrations of BA (53.25–71  $\mu\text{M}$ ) to break the latency of axillary buds and to increase the percentage of explants forming shoots up to 62%. In our laboratory, using the modified version of the method mentioned for micropropagation of *C. arabica*, a multiplication rate of seven could be achieved in 6 months by growing the cultures on MS medium with a greater concentration of 10-fold thiamine and 62  $\mu\text{M}$  BA. As reported by Custers (1980), breaking the latency of axillary buds is more effective on buds located close to apical meristem of the plant, which appears to be promising for vegetative propagation of *C. canephora* (Fig. 1D).

Another important aspect for clonal propagation of coffee is the use of mycorrhizas. Plant growth was shown to increase by 50% after inoculation with *Acaulospora melleae* or *Glomus clarum* in soils with phosphorus deficiency (Vaast et al. 1996). This approach has also been employed on plants grown under field conditions. A Brazilian group investigated the effect of endomycorrhiza, *Gigaspora margarita* (Spaggiari-Souza et al. 1991; Siqueira et al. 1994) and *G. clarum* (Siqueira et al. 1993) on the growth of *C. arabica*. Their results showed a 74% increase in productivity when superphosphates were applied in conjunction with mycorrhizal inoculation.

Optimizing the volume of nutrient medium and culture container is important for shoot proliferation when using RITA for micropropagation (Etienne and Berthouly 2002). A multiplication rate of eight for *C. arabica* can be achieved within 8 weeks using RITA, in which cultures are grown on medium containing MS salts, vitamins from Morel's medium (Morel and Wetmore 1951) and 4.43  $\mu\text{M}$  BA. The system also increases shoot vigor and improves plant quality. Hyperhydricity, which seriously affects cultures in liquid medium, can be controlled using RITA by adjusting the immersion times. Plants also perform better than those grown on semi-solid or in liquid media during acclimatization. The major advantage of RITA is the decrease in production costs owing to a drastic reduction in labor, shelving area, the number of containers used and greater productivity (Etienne and Berthouly 2002).

## 5 Genetic Transformation

Many economically important traits for crop species are controlled by the complex interaction of several genes. This may explain why crop improvement programs usually require a long period to detect and fix target traits. The use of molecular markers during the breeding process allows an increase in the number of traits that can be manipulated simultaneously (Lashermes et al. 1997).

Pests and diseases are the most important problems in coffee cultivation globally and cause considerable losses in coffee production (Omondi et al. 2001). Some important diseases include bacterial blight from *Pseudomonas syringae* pv. *garcae*, coffee berry disease induced by *Colletotrichum kahawae*, brown eye disease infected by *Cercospora coffeicola*, antracnosis from *Colletotrichum coffeanum*, and coffee leaf rust caused by *Hemileia vastatrix*. Coffee pests include coffee berry borer (*Hypothenemus hampei*), shoot borer (*Plagiohammus maculosos*) and coffee leaf miner (*Perileucoptera coffeella*).

Currently, coffee improvement relies mainly on the methods of selection based on sexual reproduction. These methods possess some limitations inherent to the species, particularly interspecific incompatibility which can hinder gene transfer from one species to another or the time (about 28 years) to release a new variety. These shortcomings may be overcome by recent advances in gene transfer technology, which allow the introduction of genes from any origin to coffee and reduce the time required for the production of novel coffee genotypes.

Genetic transformation of coffee was first achieved by electroporation of protoplasts with DNA (Acuna and de Pena 1991; Barton et al. 1991), yielding a limited number of unreliable transformed plants which took over a year to develop. Since the first report, attempts have also been made to transform coffee by co-cultivation of root explants with *Agrobacterium* spp. (Carneiro 1997). It has been reported that *C. arabica* can be transformed with wild strains of *Agrobacterium tumefaciens* (Freire et al. 1994; Hatanaka et al. 1999; Stiles et al. 1999; Perthuis et al. 2005). However, several problems associated with coffee transformation have been observed. These include low transformation efficiency, restricted transgenic plant recovery, and the regeneration of transgenic plants with no agronomically important traits.

One important factor that affects coffee transformation is the differential susceptibility of different explants and genotypes to the selective agent kanamycin. Spiral and Petiard (1993) reported that somatic embryos from *C. arabica* var. Caturra were highly tolerant to kanamycin up to 400 mg l<sup>-1</sup>, whereas germination of *C. arabica* var. Catimor somatic embryos could be inhibited in the presence of 35–50 mg l<sup>-1</sup> of kanamycin (Giménez et al. 1996). Furthermore, callus formation from leaf explants of *C. arabica* var. Catuai and *C. canephora* clone 197 could be inhibited by kanamycin at 100–200 mg l<sup>-1</sup>, but a similar inhibitory effect of kanamycin was not observed in the hybrid Arabusta. However, in *C. arabica* var. Catimor, the presence of 50 mg l<sup>-1</sup> kanamycin was sufficient to inhibit callus formation from leaf explants (Giménez et al. 1996). Apart from kanamycin, other selection agents have been studied, including chlorsulfuron, glyphosate, glyphosate and hygromycin (Van Bostel et al. 1997) and cefotaxime (Sugiyama et al. 1995). These authors recommended two selection systems for coffee transformation. The first system consisted of pretreatment of explants on callus induction medium for 1–2 days before transformation, followed by culture under the selective pressure of 1–3 mg l<sup>-1</sup> glufosinate. The other system involves pretreatment of embryogenic suspen-

sion cultures for 1 week in the regeneration medium prior to transformation, then culture in the presence of  $3 \text{ mg l}^{-1}$  glufosinate for 3–5 days. Gelling agents in the medium can also influence the activity of antibiotics (Giménez et al. 1996). The results of these studies indicate that the success of genetic transformation is affected by several factors, including the plant variety, explant source, culture medium, bacterial strains and expression vectors.

The results from several lines of study show that coffee can be transformed using various gene transfer techniques. These include electroporation of protoplasts isolated from embryogenic calli from *C. arabica* (Barton et al. 1991; Adams and Zarowitz 1994; Fernandez-Da Silva and Menéndez-Yuffá 2003), co-cultivation of torpedo-stage somatic embryos from *C. arabica* cv. Caturra Rojo, *C. canephora* and Arabusta 1307 with *A. rhizogenes* (Spiral et al. 1993; Sugiyama et al. 1995; Perthuis et al. 2005), and particle bombardment of leaf explants from *C. arabica*, *C. canephora* and Arabusta (Nagai et al. 1992; Van Boxtel et al. 1995; Ribas et al. 2005). In our laboratory, we have taken advantage of the ease of generating somatic embryos from leaf explants (Quiroz-Figueroa et al. 2002) for coffee transformation. This was conducted by vacuum infiltration of explants with *Agrobacterium*, followed by induction of SE. This approach has shown to provide a fast and reliable mean of producing transgenic coffee plants (Canché-Moor et al. 2006).

In addition to *Agrobacterium*-mediated transformation, an optimized bombardment protocol to introduce DNA into *C. arabica* cells was developed by Rosillo et al. (2003). Osmotic preconditioning of cells and physical bombardment parameters, including helium pressure, gap and target distances, were evaluated by monitoring transient expression of the *uidA* gene, which encodes  $\beta$ -glucuronidase (GUS), driven by the cauliflower mosaic virus (CaMV) 35S promoter. The transgene was shown to express transiently at the highest level in cells pre-treated with 0.5 M mannitol-sorbitol for 4 h prior to bombardment, using a helium pressure of 1,550 psi, a 9-mm gap distance and 12-cm target distance as physical bombardment parameters. The optimized protocol was tested by comparison of expression driven by the 35S and two coffee promoters,  $\alpha$ -tubulin and arabicin. Fluorometric GUS assays showed that the three promoters conferred comparable transgene expression in cell suspensions and bombarded leaves, but the former was greater than the latter. In coffee endosperm, transgene expression was detected only with 35S and arabicin promoters (Rosillo et al. 2003).

Transgenic *C. canephora* plants expressing a *cry1Ac* gene of *Bacillus thuringiensis* encoding an endotoxin, which acts against the coffee leaf miner, have been generated by co-cultivation of somatic embryos with *A. rhizogenes* or *A. tumefaciens* using chlorsulfuron as the selective agent (Leroy et al. 1997, 1999, 2000; Dufour et al. 2000; Perthuis et al. 2005). Integration of transgenes was confirmed by PCR and Southern blot analysis. However, these transgenic plants have not been evaluated for their resistance to leaf miner.

In order to regulate caffeine biosynthesis in planta, Ogita et al. (2003, 2004) suppressed expression of a gene encoding 7-*N*-methylxanthine methyltrans-

ferase (CaMXMT1) using double stranded RNA interference. Transformed embryogenic tissues of *C. arabica* and transgenic plant of *C. canephora*

showed a marked reduction in the levels of transcripts and enzyme activities of theobromine synthase, and caffeine synthase transcripts, compared to non-transformed control plants. Furthermore, both embryonic tissues and plantlets exhibited a concomitant reduction in the theobromine and caffeine content down to 30–50% of that of the control.

Although the ability to produce transgenic coffee plants is based on the availability of an efficient genetic transformation, the success in genetic transformation relies mainly on an efficient tissue culture system for plant regeneration from cultured cells and tissues. Transgenic plants are important for coffee improvement through gene transfer and expression of novel genes responsible for agronomic or industrially important traits. They also serve as an important tool for the study of gene function and regulation, leading to an improved understanding of the fundamental aspects of coffee biology at the molecular level.

## 6 Conclusions

Coffee is an important commercial crop that has not been studied extensively. Genetic improvement of this crop is slow owing, at least in part, to its perennial nature. Biotechnology has emerged as an alternative tool for generating new coffee varieties in shorter periods of time and for rapid clonal propagation of economically important coffee varieties. Although tissue culture methods for scaling up and multiplication of elite cultivars have been reported, further improvement of these methods is needed to increase efficiency. Among the biotechnological tools, SE and molecular markers have the most potential. SE can be used to multiply F1 hybrids from breeding programs. At the same time, molecular markers allow the selection of plants with desirable traits. Techniques such as amplified fragment length polymorphism also allow the identification of unknown varieties or the introgression of genes in new hybrids. Genetic engineering will provide low-caffeine coffee, uniform fruit maturation and, hence, mechanized harvest and strict control over seasonality, as well as coffee varieties resistant to environmental extremes, pests and diseases.

It is reasonable to expect that future coffee plantations may comprise new varieties generated from biotechnological approaches such as genetic engineering, protoplast fusion, embryo rescue, somaclonal variation or haploid culture. These new varieties can be multiplied by SE in large-scale systems and identified, classified and selected by means of molecular markers. Thus coffee for the 21st century may be produced using biotechnological approaches in combination with traditional agricultural techniques.

## References

- Acuna JR, de Pena M (1991) Plant regeneration from protoplasts of embryogenic cell suspensions of *Coffea arabica* L. cv. caturra. *Plant Cell Rep* 10:345–348
- Adams TL, Zarowitz MA (1994) Stably transformed coffee plant cells and plantlets. Appl no. 988009, Pat no. 5334529, USA, 1–8 (Patent)
- Albarrán J, Bertrand B, Lartaud M, Etienne H (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. *Plant Cell Tissue Organ Cult* 81:27–36
- Aponte AME (1993) Somatic embryogenesis induced by culture on single media in coffee plant crosses of *Coffea arabica* by timor hybrid. In: *Proc 15th Colloque Scientifique International sur le Café*. Association Scientifique Internationale du Café, Paris, pp 82–88
- Ascanio ECE, Arcia MM (1987) Haploids from anther culture in *Coffea arabica* L. In: *Proc Int Congr on Plant Tissue Culture, Tropical Species*, Bogotá
- Ascanio ECE, Arcia MMA (1994) Efecto del estado de desarrollo de las anteras y de un shock térmico sobre la androgénesis en *Coffea arabica* L. var. Garnica. *Café Cacao Thé* 38:75–80
- Barton CR, Adams TL, Zarowitz MA (1991) Stable transformation of foreign DNS into *Coffea arabica* plants. In: *Proc 14th Colloque Scientifique Internationale sur le Café*, Association Scientifique Internationale du Café, Paris, pp 460–464
- Berthouly M, Dufour M, Alvard D, Carasco C, Alemanno L, Teisson C (1995) Coffee micropropagation in a liquid medium using the temporary immersion technique. In: *Proc 16th Colloque Scientifique International sur le Café*, Association Scientifique Internationale du Café, Paris, pp 514–519
- Berthouly M, Michaux-Ferrière N (1996) High frequency somatic embryogenesis in *Coffea canephora*. Induction conditions and histological evolution. *Plant Cell Tissue Organ Cult* 44:169–176
- Biesse D, Gofflot A, Michaux-Ferrière N (1993) Effect of experimental conditions and genotypic variability on somatic embryogenesis in *Coffea arabica*. *Can J Bot* 71:1496–1502
- Calheiros MBP, Vieira LGE, Fuentes SRL (1994) Effects of exogenous polyamines on direct somatic embryogenesis in coffee. *R Bras Fisiol Veg* 6:109–114
- Canché-Moor RLR, Kú-González A, Burgeff C, Loyola-Vargas VM, Rodríguez-Zapata LC, Castaño E (2006) Biotechnology and its applications to coffee improvement. *Plant Cell Tissue Organ Cult* 84:373–377
- Carneiro MF (1997) Coffee biotechnology and its application in genetic transformation. *Euphytica* 96:167–172
- Carneiro MF (1999) Advances in coffee biotechnology. *AgBiotechNet* 1:1–7
- Chatterjee G, Singh G, Thangam P (1991) Clonal propagation of bamboo, coffee and mimosa. *Curr Plant Sci Biotechnol Agric* 261–264
- Cid LPB, Cruz ARR, Castro LHR (2004) Somatic embryogenesis from three coffee cultivars: ‘Rubi’, ‘Catuai Vermelho 81’, and ‘IAPAR 599’. *HortScience* 39:130–131
- Custers JBM (1980) Clonal propagation of *Coffea arabica* L. by nodal culture. In: *Proc 9th Colloque Scientifique Internationale sur le Café*, Association Scientifique Internationale du Café, Paris, pp 588–596
- De Fera M, Jiménez E, Barbón R, Capote A, Chávez M, Quiala E (2003) Effect of dissolved oxygen concentration on differentiation of somatic embryos of *Coffea arabica* cv. Catimor 9722. *Plant Cell Tissue Organ Cult* 72:1–6
- de García E, Menéndez A (1987) Embriogénesis somática a partir de explantes foliares del cafeto ‘Catimor’. *Café Cacao Thé* 31:15–22
- de García E, Rafael M (1989) Propagación clonal de plantas de café (*Coffea arabica* L. “Catimor”) a partir de microesquejes cultivados in vitro. *Agron Trop* 39:249–268
- Dublin P (1980a) Induction de bourgeons néoformés et embryogénèse somatique. Deux voies de multiplication végétative in vitro des caféiers cultivés. *Café Cacao Thé* 29:121–130
- Dublin P (1980b) Multiplication végétative in vitro de l’Arabusta. *Café Cacao Thé* 29:281–290



- Dublin P (1981) Embryogenèse somatique directe sur fragments de feuilles de caféier Arabusta. *Café Cacao Thé* 25:237–242
- Dublin P (1984) Techniques de reproduction végétative in vitro et amélioration génétique chez les caféiers cultivés. *Café Cacao Thé* 28:231–244
- Ducos JP, Zamarripa CA, Eskes AB, Pétiard V (1993) Production of somatic embryos of coffee in a bioreactor. In: Proc 15th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café, Paris, pp 89–96
- Ducos JP, Alenton R, Reano JF, Kanchanomai C, Deshayes A, Tiard V (2003) Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. *Euphytica* 131:215–223
- Dufour M, Leroy T, Carasco-Lacombe C, Philippe R, Fenouillet C (2000) Coffee (*Coffea* sp.) genetic transformation for insect resistance. In: Sera T, Soccol CR, Pandey A, Roussos S (eds) *Coffee biotechnology and quality*. Kluwer, Dordrecht, pp 209–217
- Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. *Plant Cell Tissue Organ Cult* 69:215–231
- Etienne H, Bertrand B, Anthony F, Cote F, Berthouly M (1997) L'embryogenèse somatique: un outil pour l'amélioration génétique du caféier. Association Scientifique Internationale du Café, Paris, pp 457–465
- Etienne-Barry D, Bertrand B, Vasquez N, Etienne H (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. *Plant Cell Rep* 19:111–117
- Fernandez-Da Silva R, Menéndez-Yuffá A (2003) Transient gene expression in secondary somatic embryos from coffee tissues electroporated with the genes *gus* and *bar*. *Elect J Biotechnol* 6: 29–38
- Freire V, Lightfoot DA, Preece JE (1994) Genetic transformation of coffee (*Coffea arabica* L.) by *Agrobacterium* spp. *HortScience* 29 (Abstract):454
- Fuentes SRL, Calheiros MBP, Manetti J, Vieira LGE (2000) The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell Tissue Organ Cult* 60:5–13
- Fuentes-Cerda CFJ, Monforte-González M, Méndez-Zeel M, Rojas-Herrera R, Loyola-Vargas VM (2001) Modification of the embryogenic response of *Coffea arabica* by nitrogen source. *Biotechnol Lett* 23:1341–1343
- Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50:151–158
- Gavish H, Vardi A, Fluhr R (1992) Suppression of somatic embryogenesis in *Citrus* cell cultures by extracellular proteins. *Planta* 186:511–517
- Giménez ACA, Menéndez-Yuffá A, García E (1996) Efecto del antibiótico kanamicina sobre diferentes explantes del híbrido de café *Coffea* sp. Catimor. *Phyton* 59:39–46
- Giridhar P, Indu EP, Ravishankar GA, Chandrasekar A (2004a) Influence of triacontanol on somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P. ex Fr. *In Vitro Cell Dev Biol-Plant* 40:200–203
- Giridhar P, Indu EP, Vinod K, Chandrasekar A, Ravishankar GA (2004b) Direct somatic embryogenesis from *Coffea arabica* L. and *Coffea canephora* P ex Fr. under the influence of ethylene action inhibitor-silver nitrate. *Acta Physiol Plant* 26:299–305
- Gresshoff PM, Doy CH (1972) Haploid *Arabidopsis thaliana* callus and plants from anther culture. *Aust J Biol Sci* 25:259–264
- Grèzes J, Thomas D, Thomasset B (1994) Factors influencing protoplast isolation from *Coffea arabica* cells. *Plant Cell Tissue Organ Cult* 36:91–97
- Halperin W, Wetherell DF (1965) Ammonium requirement for embryogenesis in vitro. *Nature* 205:519–520
- Hatanaka T, Arakawa O, Yasuda T, Uchida N, Yamaguchi T (1991) Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Rep* 10:179–182
- Hatanaka T, Sawabe E, Azuma T, Uchida N, Yasuda T (1995) The role of ethylene in somatic embryogenesis from leaf disks of *Coffea canephora*. *Plant Sci* 107:199–204



- Hatanaka T, Choi YE, Kusano T, Sano H (1999) Transgenic plants of coffee *Coffea canephora* from embryogenic callus via *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Rep* 19:106–110
- Heller R (1953) Recherches sur la nutrition minerale des tissus vegetaux in vitro. *Ann Sci Natl Bot* 14:1–10
- Herman EB, Haas GJ (1975) Clonal propagation of *Coffea arabica* L. from callus culture. *HortScience* 10:588–589
- Kao KN, Michayluk R (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta* 126:1095–1100
- Lanaud C (1981) Production of *Coffea canephora* plantlets by somatic embryogenesis obtained by in vitro culture of ovules. *Café Cacao Thé* 25:231–236
- Lashermes P, Agwanda CO, Anthony F, Combes MC, Trouslot P, Charrier A (1997) Molecular marker-assisted selection: a powerful approach for coffee improvement. In: *Proc 17th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 474–480
- Leroy T, Paillard M, Berthouly M, Spiral J, Tessereau S, Legavre T, Altosaar I (1997) Introduction de gènes d'intérêt agronomique dans l'espèce *Coffea canephora* Pierre part transformation avec *Agrobacterium* sp. In: *Proc 17th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 439–446
- Leroy T, Philippe R, Royer M, Frutos R, Duris D, Dufour M, Jourdan I, Lacombe C, Fenouillet C (1999) Genetically modified coffee trees for resistance to coffee leaf miner. Analysis of gene expression, resistance to insects and agronomic value. In: *Proc 18th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 332–338
- Leroy T, Henry AM, Royer M, Altosaar I, Frutos R, Duris D, Philippe R (2000) Genetically modified coffee plants expressing the *Bacillus thuringiensis cryIIAc* gene for resistance to leaf miner. *Plant Cell Rep* 19:382–389
- Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* 18:100–127
- Marques DV (1987) Study of some factors involved on in vitro callus growth and somatic embryogenesis of coffee tissues. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) *Plant biology*, vol 3. Plant tissue and cell culture. Alan R Liss, New York
- Mattern U, Strobel G, Shepard J (1978) Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. *Proc Natl Acad Sci (USA)* 75:4935–4939
- Mazzafera P, Carvalho A (1992) Breeding for low caffeine content of coffee (*Coffea* L.) by inter-specific hybridization. *Euphytica* 59:55–60
- Michaux-Ferrière N, Bieysse D, Alvard D, Dublin P (1989) Étude histologique del l'embryogenèse somatique chez *Coffea arabica*, induite par culture sur milieux uniques de fragments foliaires de génotypes différents. *Café Cacao Thé* 33:207–217
- Molina DM, Aponte ME, Cortina H, Moreno G (2002) The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant Cell Tissue Organ Cult* 71:117–123
- Möllers C, Zitzlsperger J, Wenzel G (1992) The effects of a toxin preparation from *Phytophthora infestans* on potato protoplasts and microspores. *Physiol Mol Plant Pathol* 41:427–435
- Monaco LC, Söndahl MR, Carvalho A, Crocomo OJ, Sharp WR (1977) Application of tissue culture in the improvement of coffee. In: Reinhard E, Bajaj YPS (eds) *Applied and fundamental aspects of plant cell, tissue, and organ culture*. Springer, Berlin Heidelberg New York, pp 109–129
- Montes S, Martínez M, Rojas R, Santana N, Cuba M (1995) Obtención de embriones somáticos a partir de suspensiones celulares de *Coffea canephora* variedad Robusta. *Cultivos Tropicales* 16:77–81
- Morel G, Wetmore RH (1951) Tissue culture of monocotyledons. *Am J Bot* 38:138–140
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nagai C, Mai Z, Jong J (1992) Development of transformation system for *Coffea arabica* using particle gun method. *HortScience* 27 (Abstract):661

- Nassuth A, Wormer TM, Bouman F, Staritsky G (1980) The histogenesis of callus in *Coffea canephora* stem explants and the discovery of early embryoid initiation. *Acta Bot Neerl* 29:49–54
- Neuenschwander B, Baumann TW (1992) A novel type of somatic embryogenesis in *Coffea arabica*. *Plant Cell Rep* 10:608–612
- Neuenschwander B, Dufour M, Baumann TW (1993) Haploid cell colony formation from mechanically isolated microspores of *Coffea arabica*. In: *Proc 15th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 760–762
- Neuhaus G, Spangenberg G, Mittelsten Scheid O, Schweiger H-G (1998) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. *Theor Appl Genet* 75:30–36
- Nishibata T, Azuma T, Uchida N, Yasuda T, Yamaguchi T (1995) Amino acids on somatic embryogenesis in *Coffea arabica*. In: *Proc 16th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 839–844
- Nyange NE, McNicol R, Williamson B (1997a) Plant regeneration from suspension culture protoplasts established from hypocotyl-derived callus of two *Coffea arabica* genotypes. In: *Proc 17th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café*, Paris, pp 779–782
- Nyange NE, Williamson B, Lyon GD, McNicol RJ, Connolly T (1997b) Responses of cells and protoplasts of *Coffea arabica* genotypes to partially purified culture filtrates produced by *Colletotrichum kahawae*. *Plant Cell Rep* 16:763–769
- Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) RNA interference: producing decaffeinated coffee plants. *Nature* 423:823
- Ogita S, Uefuji H, Morimoto M, Sano H (2004) Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. *Plant Mol Biol* 54:931–941
- Omondi CO, Ayiecho PO, Mwang'ombe AW, Hindorf H (2001) Resistance of *Coffea arabica* cv. ruiru 11 tested with different isolates of *Colletotrichum kahawae*, the causal agent of coffee berry disease. *Euphytica* 121:19–24
- Orozco FJ, Schieder O (1982) Aislamiento y cultivo de protoplastos a partir de hojas de café. *Cenicafé* 33:129–136
- Orozco FJ, Schieder O (1984) Isolation of mesophyll protoplasts of the genus *Coffea*. *Turrialba* 34:534–536
- Owuor JBO (1987) In vitro initiation of Arabusta coffee hybrids. *Kenia Coffee* 52:59–62
- Perthuis B, Pradon J, Montagnon C, Dufour M, Leroy T (2005) Stable resistance against the leaf miner *Leucoptera coffeella* expressed by genetically transformed *Coffea canephora* in a pluriannual field experiment in French Guiana. *Euphytica* 144:321–329
- Quiroz-Figueroa FR, Méndez-Zeel M, Larqué-Saavedra A, Loyola-Vargas VM (2001) Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture. *Plant Cell Rep* 20:679–684
- Quiroz-Figueroa FR, Fuentes-Cerda CFJ, Rojas-Herrera R, Loyola-Vargas VM (2002) Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. *Plant Cell Rep* 20:1141–1149
- Raghuramulu Y (1989) Anther and endosperm culture of coffee. *J Coffee Res* 19:71–81
- Raghuramulu Y, Purushotham K, Sreenivasan MS, Ramaiah PK (1987) In vitro regeneration of *Coffea* plantlets in India. *J Coffee Res* 17:57–64
- Ribas AF, Kobayashi AK, Pereira LFP, Vieira LGE (2005) Genetic transformation of *Coffea canephora* by particle bombardment. *Biol Plant* 49:493–497
- Rosillo AG, Acuna JR, Gaitán AL, de Pena M (2003) Optimised DNA delivery into *Coffea arabica* suspension culture cells by particle bombardment. *Plant Cell Tissue Organ Cult* 74:45–49
- Santana N (1993) Embriogénesis somática en el cultivo del cafeto (*Coffea* sp.). PhD thesis, Instituto Nacional de Ciencias Agrícolas, La Habana, pp 1–154
- Santana N, Martínez O, Gonzales MC (1988) Embriogénesis somática en el cultivo del café (*Coffea arabica*). (Parte I.) *Cultivos Tropicales* 10:36–43

- Santana-Buzzy N, González ME, Valcárcel M, Canto-Flick A, Barzaga ML, Hernández MM, Fuentes-Cerda CFJ, Barahona F, Mijangos-Cortés J, Loyola-Vargas VM (2004) Somatic embryogenesis: a valuable alternative to propagate selected robusta (*Coffea canephora*) clones. In *Vitro Cell Dev Biol-Plant* 40:95–101
- Schöpke C, Mueller LE, Kohlenbach HW (1987) Somatic embryogenesis and regeneration of plantlets in protoplast cultures from somatic embryos of coffee (*Coffea canephora* P. ex Fr.). *Plant Cell Tissue Organ Cult* 8:243–248
- Sharp WR, Caldas LS, Crocomo OJ, Monaco LC, Carvalho A (1973) Production of *Coffea arabica* callus of three ploidy levels and subsequent morphogenesis. *Phyton* 31:67–74
- Shepard JF, Bidney D, Shahin E (1980) Potato protoplasts in crop improvement. *Science* 208:17–24
- Siqueira JO, Colozzi-Filho A, Saggin-Júnior OJ, Guimaraes PTG, Oliveira E (1993) Comissao III – biologia do solo. *R Bras Ci Solo Campinas* 17:53–60
- Siqueira JO, Colozzi-Filho A, Saggin-Júnior OJ (1994) Efeito da infeccao de plantulas de cafeeiro com quantidades crescentes de esporos do fungo endomicorrizico *Gigaspora margarita*. *Pesq Agropec Bras Brasilia* 29:875–883
- Söndahl MR, Noriega C (1992) Coffee somatic embryogenesis in liquid cultures. In *Vitro Cell Dev Biol-Plant* 28 (Abstract):93A
- Söndahl MR, Sharp WR (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Z Pflanzenphysiol* 81:395–408
- Söndahl MR, Spahlinger D, Sharp WR (1979) A histological study of high frequency and low frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Z Pflanzenphysiol* 94:101–108
- Söndahl MR, Chapman MS, Sharp WR (1980) Protoplast liberation, cell wall reconstitution, and callus proliferation in *Coffea arabica* L. callus tissues. *Turrialba* 30:161–165
- Söndahl MR, Monaco LC, Sharp WR (1981) In vitro methods applied to coffee. In: Thorpe TA (ed) *Plant tissue culture. Methods and applications in agriculture*. Academic Press, New York, pp 325–347
- Söndahl MR, Nakamura T, Sharp WR (1985) Propagation of coffee. *Basic Life Sci* 32:215–232
- Spaggiari-Souza CA, Siqueira JO, De Oliveira E, De Carvalho JG (1991) Crescimento e nutricao de mudas de cafeeiro micorrizadas. Efeito da matéria orgânica e superfosfato simples. *Pesq Agropec Bras Brasilia* 26:1989–2005
- Spiral J, Pétiard V (1991) Protoplast culture and regeneration in *Coffea* species. In: *Proc 14th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café, Paris*, pp 383–391
- Spiral J, Pétiard V (1993) Développement d'une méthode de transformation appliquée à différentes espèces de caféier et régénération de plantules transgéniques. In: *Proc 15th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café, Paris*, pp 115–122
- Spiral J, Thierry C, Paillard M, Petiard V (1993) Regeneration of plantlets of *Coffea canephora* Pierre (Robusta) transformed by *Agrobacterium rhizogenes*. *C R Acad Sci (Paris) Sér III* 316:1–6
- Sreenath HL, Shanta HM, Babu KH, Naidu MM (1995) Somatic embryogenesis from integument (perisperm) cultures of coffee. *Plant Cell Rep* 14:670–673
- Staritsky G (1970) Embryoid formation in callus tissues of coffee. *Acta Bot Neod* 19:509–514
- Stiles JJ, Moisyadi I, Neupane KR (1999) Purified proteins, recombinant DNA sequences and processes for controlling the ripening of coffee plant. Appl no. 695412, Pat no. 5874269, USA, 1–30 (Patent)
- Sugiyama M, Matsuoaka C, Takagi T (1995) Transformation of coffee with *Agrobacterium rhizogenes*. In: *Proc 16th Colloque Scientifique Internationale sur le Café, Association Scientifique Internationale du Café, Paris*, pp 853–859
- Tahara M, Yasuda T, Uchida N, Yamaguchi T (1994) Formation of somatic embryos from protoplasts of *Coffea arabica* L. *HortScience* 29:172–174
- Toruan-Mathius N (1992) Isolation and protoplasts culture of *Coffea arabica* L. *Biotechnol Forest Tree Improvement* 49:89–98

- Vaast Ph, Zasoski RJ, Bledsoe CS (1996) Effects of vesicular-arbuscular mycorrhizal inoculation at different soil P availabilities on growth and nutrient uptake of in vitro propagated coffee (*Coffea arabica* L.) plants. *Mycorrhiza* 6:493–497
- Van Bostel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. Factors influencing embryogenesis, and subsequent proliferation and regeneration in liquid medium. *Plant Cell Tissue Organ Cult* 44:7–17
- Van Bostel J, Berthouly M, Carasco C, Dufour M, Eskes A (1995) Transient expression of b-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. *Plant Cell Rep* 14:748–752
- Van Bostel J, Eskes AB, Berthouly M (1997) Glufosinate as an efficient inhibitor of callus proliferation in coffee tissue. *In Vitro Cell Dev Biol-Plant* 33:6–12
- Wenzel G, Foroughi-Wehr B (1990) Progeny tests of barley, wheat and potato regenerated from cell cultures after in vitro selection for disease resistance. *Theor Appl Genet* 80:359–365
- Yasuda T, Fujii Y, Yamaguchi T (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. *Plant Cell Physiol* 26:595–597
- Yasuda T, Tahara M, Uchida N, Yamaguchi T (1987) Somatic embryogenesis from coffee callus and protoplast. In: Green CE, Somers DA, Hackett WP, Biesboer DD (eds) *Plant biology*, vol 3. *Plant tissue and cell culture*. Alan R Liss, New York
- Zamarripa CA, Ducos JP, Bollon H, Dufour M, Petiard V (1991a) Production d'embryons somatiques de caféier en milieu liquide: effets densité d'inoculation et renouvellement du milieu. *Café Cacao Thé* 35:233–244
- Zamarripa CA, Ducos JP, Tessereau H, Bollon H, Eskes AB, Pétiard V (1991b) Développement d'un procédé de multiplication en masse du caféier par embryogenèse somatique en milieu liquide. In: *Proc 14th Colloque Scientifique Internationale sur le Café*, Association Scientifique Internationale du Café, Paris, pp 392–402

## III.2 Cacao

M.J. GUILTINAN<sup>1</sup>

### 1 Introduction

*Theobroma cacao* L. (cacao) is a small under-story tree endemic to the lowland rainforests of the Amazon basin (Wood and Lass 1985; Bartley 2005). Cacao was domesticated in pre-Columbian times by the Olmec and Maya civilizations, the latter of which used the seeds ('cocoa beans') to produce beverages for royalty and religious ceremonies, and as currency (Coe and Coe 1996; Motamayor et al. 2002; Emch 2003). Today, cacao is grown throughout the humid tropics, often in agroforestry-ecosystems with other fruit and commodity crops.

#### 1.1 Worldwide Market

The ability to store dried cocoa beans for long periods of time and the strong demand for the production of chocolate make cacao one of the world's most valuable commodity crops. The annual world production of cocoa is approximately 3 million tons, with two-thirds being processed into cocoa powder and cocoa butter, and the remaining one-third used for cocoa liquor (the flavor and color component of chocolate) (Wood and Lass 1985). Cocoa is the major export commodity of several countries in West Africa (making up 68% of world production) and provides major economic resources to the Ivory Coast, Cameroon, Nigeria and Ghana. Other major cocoa exporters include Ecuador, Venezuela, Brazil, Panama, Costa Rica, Malaysia and Indonesia. More recently, cacao has become a priority crop in regions of Vietnam and India. It is also an important replacement cash crop for illegal narcotics in Colombia and Peru, offering farmers a legal alternative for their livelihoods. Worldwide, approximately 5–6 million smallholder farmers grow 95% of the world's production on more than 7 million ha, providing an important source of income for them and their families. World cocoa exports amount to US\$5–6 billion/year and the use of cocoa and cocoa butter in chocolate manufacturing, cosmetics and other products drives an approximately US\$70 billion market, providing over 60,000 jobs in the US alone (Morais 2005). US chocolate production also uses large amounts of sugar, nuts and milk, valued at approx. US\$3 billion/year in receipts to American farmers and similar benefits to many other countries.

---

<sup>1</sup> Department of Horticulture, 422 Life Sciences Building, The Pennsylvania State University, University Park, Pennsylvania 16802, USA, e-mail: mjpg9@psu.edu

## 1.2 Sustainability and Ecological Benefits of Cacao

Cacao-growing regions are largely centered in important biodiversity hotspots, impacting 13 of the world's most biologically diverse regions (Piasentin and Klare-Repnik 2004). For example, southern Bahia, Brazil, a major cacao-producing region, contains some of the last remaining vestiges of the Atlantic Rainforest ('Mata Atlântica'). The Ivory Coast and Ghana, world leaders in cocoa production, are both located in the Upper Guinea Forest, which contains more than half of the mammalian species in Africa, the highest mammalian diversity density on earth, many of which are endangered. Because cacao is a shade-grown perennial tree crop, with a cropping cycle of more than 50 years, cultivation creates environmental benefits such as enhancement of biodiversity in avian migratory corridors, conservation of soils and watersheds and provision of buffer zones near endangered rainforest habitats (Rice and Greenberg 2003; Ruf and Zadi 2003). Development agencies, such as USAID, Conservation International (CI), The World Wildlife Federation (WWF) and the World Cocoa Foundation, are increasingly aware of the role of cacao in stabilizing local economies and environments, and have stepped up their involvement with cacao farmers in these regions (Guyton et al. 2003).

## 1.3 Economic/Social Impacts of Cacao Diseases

Cacao pathogens reduce the potential crop by an estimated 810,000 tons annually (30% of world production) and individual farm losses can approach 100% (Keane 1992; Bowers et al. 2001). For example, in southern Bahia, Brazil, witches' broom disease (caused by the fungus *Crinipellis pernicioso*) resulted in a decrease in production from 300,000 tons in 1989 to 130,000 tons 10 years later, at an estimated loss of US\$220 million each year (Pereira et al. 1990). This devastating disease has resulted in an economic and ecological disaster, resulting in widespread homelessness and a major social crisis. Widely dispersed cacao pathogens include several species of *Phytophthora* that cause multiple diseases of economic importance, including pod rot, trunk canker and leaf-blight (Appiah et al. 2003, 2004; Chowdappa et al. 2003). *Phytophthora megakarya*, the most aggressive and damaging species, has been reported to have reached the Ivory Coast, the world's leading cocoa producer (Nyasse et al. 2002; Opoku et al. 2002; Appiah et al. 2003; Risterucci et al. 2003; Efombagn et al. 2004). Other important diseases and pests include frosty pod in Central America (Evans et al. 2003), the cocoa pod borer in Asia (Day 1984; Santoso et al. 2004) and cocoa swollen shoot virus, which is particularly devastating in Ghana, Africa, and is also spreading to the Ivory Coast (Hanna 1954; Hervé et al. 1991; Muller and Sackey 2005).

It is estimated that only 30% of the varieties grown today are the result of selection programs, and the remaining 70% are introductions of non-selected plants with low disease resistance and yield potential, originally derived from



the Amazon basin and propagated by farmers through seeds passed down through the generations (Bennett 2003). While large genetic variation has been identified in wild populations throughout the Amazon, this diversity has not been widely utilized in terms of practical incorporation into cultivated varieties (Bartley 2005). This dramatically illustrates the primitive state of domestication of cacao, but it also highlights the opportunities for future enhancement through the application of modern molecular/genetic breeding approaches. It is widely accepted that although agronomic and agrochemical technologies can play important roles in integrated pest management strategies for cacao, breeding has the advantage of delivering genetically encoded traits such as disease resistance to the farmer, so that fewer inputs are required once the trees have been planted. This is especially important since cacao is often grown by poor farmers who cannot afford expensive agricultural inputs. Disease resistance is currently the primary trait targeted by cacao breeders. Other important traits for cacao include yield efficiency, flavor characteristics, cocoa butter content (percentage seed lipid content) and quality (fatty acid saturation), tolerance to abiotic stress, and various horticultural traits such as precocity, rootstock/scion interactions, plant height and stature.

#### **1.4 Cacao Molecular Genetics Research Community**

Today's cacao genetics research community is well organized, highly collaborative and poised to make use of new genomics resources, which has been reviewed by Bennett (2003). In order to formally foster collaboration and communication between cacao breeders and geneticists, the International Group for Genetic Improvement of Cocoa (INGENIC) was formed in 1994. It now includes over 300 members, representing 35 developing and developed countries around the world. The INGENIC Study Group for Molecular Biology (INGENIC-MOL-BIOL) was formally chartered in October 2003 (Johnson 2003) to coordinate the activities of the INGENIC members interested in molecular approaches. An international research symposium is held by INGENIC in a developing country every third year. More information about INGENIC is available at the website (<http://ingenic.cas.psu.edu>).

In addition to cacao research centers in developed countries such as the US, France and the UK, most of the cocoa-producing countries have research facilities funded by international and national organizations that support agricultural research. The sophistication of these facilities varies from elementary to excellent. The main strengths of these organizations are the many scientists with strong experiences in cacao agriculture, as well as their extensive field sites and breeding programs. It is essential that researchers in developed and developing countries establish and maintain strong working relationships and collaborative research and training programs to maximize the potential impact of our research on cacao farmers and the environment.



## 2 Recent Advances: A Recent Literature Review

### 2.1 Molecular Markers

#### 2.1.1 Germplasm Evaluation via DNA Fingerprinting

One of the central applications of molecular biology to plant genetics is the use of molecular markers to determine the relationships between accessions using phylogenetic analysis. For cacao, this has been approached with isozymes, random amplified polymorphic DNA (RADP), restriction fragment length polymorphisms (RFLP) and other types of genomic DNA markers. Most recently, microsatellite markers (simple sequence repeats – SSRs) have gained acceptance as the most accurate and reliable method for fingerprinting germplasm to identify them and to find mislabelling and mistakes (Atkinson et al. 1986; Fritz et al. 1991; Wilde et al. 1991; Figueira et al. 1994; Ronning et al. 1995; Lerceteau et al. 1997; Crouzillat et al. 2000; Gomes et al. 2000; Niella et al. 2000; Dias 2001; Motamayor et al. 2002; Kuhn et al. 2003; Borrone et al. 2004; Faleiro et al. 2004; Pugh et al. 2004; Brown et al. 2005; Sereno et al. 2006). Using these approaches, our understanding of the evolutionary relationships between different cacao accessions and of the relationships between members of germplasm collections has advanced rapidly (Figueira et al. 1994; Whitkus et al. 1998; Marita et al. 2001; Motamayor et al. 2002). These investigations have ranged from characterizing germplasm collections, to elucidating the origin of different cacao lineages spanning back to the ancient Mayas. For example, molecular markers have been used to shed light on a long-standing controversy regarding the origins of a particular lineage of cacao, the *Criollo*, believed to have been cultivated by the Mayas over 1500 years ago (Motamayor et al. 2002, 2003). This analysis suggests that contrary to the hypothesis put forward by others, *Criollo cacao* does not represent a separate subspecies, but may have derived from a few individuals in South America and then later was spread by man into Central America. As discussed in the epilogue of his recent book, Bartley (2005) points out that this indicates that the *Criollo* group represents an insignificant proportion of the total diversity in the species. Understanding these lineages has important implications to breeders who wish to incorporate diverse sources of resistance and other traits into breeding programs.

In a broader sense, the same methodology can be used to investigate the evolutionary relationships of cacao with other species. In this way, using molecular phylogenetic analysis of chloroplast DNA sequences, *Theobroma cacao* and its related species have been reclassified from the family Sterculiaceae to the Malvaceae (Alverson et al. 1999; Bayer et al. 1999; Whitlock et al. 2001). This is important to cacao researchers who wish to extrapolate information from other related species, such as cotton, which is in the same family as cacao, and *Arabidopsis thaliana*, a model plant for molecular-genetic research worldwide that is also closely related to cacao.

Cacao germplasm collections are found in several sites throughout the world, and the diversity maintained in these collections is an important resource for breeders. These accessions are systematically being evaluated for various traits of interest, such as disease resistance and quality (Iwaro et al. 2003). One of the clear messages from the germplasm characterization work has been the realization that many of the genotypes residing in collections are mislabeled and that DNA markers can be used to fingerprint accessions and rectify some of these discrepancies (Charters and Wilkinson 2000). International standards for cacao fingerprinting have been agreed upon and are being adjusted and modified by the cacao research community through mutual cooperation and testing (Saunders et al. 2004; Cryer et al. 2006). With these new standards, germplasm collections are now being systematically characterized, and mislabeling problems corrected. Cryer et al. (2006) described two methods of standardization of microsatellite allele profiles between different laboratories. This was performed on a total of 429 cacao accessions. These authors fingerprinted these accessions with 15 microsatellite markers that have been agreed upon as a common set for worldwide standardization. Importantly, the data were deposited in the International Cocoa Germplasm Database, which is an easily accessible and searchable database for cacao molecular genetic data, and another new important resource for the cacao research community (<http://www.icgd.rdg.ac.uk>).

An excellent example of how molecular markers can be used to study the genetic diversity and population structure of cacao collections was published recently (Serenio et al. 2006). Four populations from the upper and lower Amazon were analyzed using microsatellite makers. Interestingly, the accessions from the upper Amazon contained the largest diversity, and thus the authors suggest that this specific sub-region might be the center of diversity for cacao. Another example of using fingerprinting was published by Faleiro et al. (2004), who studied the genetic variability of 19 accessions from around the Amazon basin. RAPD and microsatellite markers were used to demonstrate the high genetic variability of this collection, which is not surprising considering the Amazon is the center of origin for *Theobroma cacao* (Cruz et al. 1995; Motamayor et al. 2002, 2003).

Individual gene sequences from different accessions can also be used to study genetic diversity. Sousa Silva and Figueira (2005) published a study using the sequences of the Kunitz-like trypsin inhibitor gene from 11 *Theobroma* and three related *Herrania* species. The phylogenetic analysis carried out using these sequences was consistent with the phylogeny previously proposed based on morphological characters.

One aspect of developing molecular makers for germplasm characterization and marker-assisted selection is to generate highly reproducible and economical methods that can be applied in a large scale in the future. Kuhn et al. (2003, 2005) and Kuhn and Schnell (2005) reported on the development of new genetic markers for cacao using a technique, capillary array electrophoresis-single strand conformation polymorphism (CAE-SSCP), which has never be-

fore been applied to cacao. This method is capable of detecting single base pair differences between two alleles of a gene, and multiple markers can be multiplexed in single reaction tubes. Using automated sample handling and high throughput automated DNA fragment detection systems, a very high throughput system was developed. The authors showed that CAE-SSCP was capable of detecting single nucleotide polymorphisms in cacao, and specified parameters to increase the reliability of the method. If this method is adopted internationally, it could accelerate mapping and fingerprinting efforts.

Molecular markers can also be used to study mutation rates in cells, as was recently reported (Rodriguez Lopez et al. 2004). In this study, simple sequence repeat (SSR) analysis was used to detect mutations in *in vitro* grown plantlets derived from cacao somatic embryos. Analysis of 233 regenerated plants revealed that 31% of the plants contained putative chimeric mutations, that is to say, portions of the plants appeared to have mutations. The plants tested were not regenerated and acclimated into glasshouse conditions, a process that is known to select against undesirable mutations from populations of embryos. It will be interesting to see if this result is specific for the population of plants tested in this study, or is representative of *in vitro* cacao plants regenerated in other labs or with different protocols.

### *2.1.2 Molecular Mapping*

Recent advances in the field of cacao genetic mapping and breeding have been reviewed recently (Bennett 2003; Figueira and Alemanno 2005). Much of this work is performed in collaborative projects between laboratories in developed and in producing countries. Recent progress has involved the development of useful markers for mapping, the production of a reference molecular-genetic map and the application of these markers in quantitative trait loci (QTL) mapping studies. One landmark study was the creation of a reference molecular-genetic map of cacao, which was developed by the Lanaud group in France (Risterucci et al. 2000; Pugh et al. 2004). This map currently consists of >250 SSRs and >400 RFLP, RAPD, AFLP and isozyme markers, covering ~900 cM of the 10 cacao chromosomes with an average marker distance of ~2 cM. This framework map is important as it serves as a scaffold for genetics and genomics research in the future, and will allow the integration of QTL maps from different genotypes for various traits. Another landmark has been the establishment of a large number of microsatellite markers for mapping in cacao (Lanaud et al. 1999; Pugh et al. 2004). These authors created a specific genomic DNA sub-library enriched with short repetitive DNA elements (microsatellite DNA), which are very useful as molecular markers because the length of the repeat elements is highly variable between genotypes and easy to measure using conventional PCR-based analysis.

Quantitative trait loci (QTL) mapping is a technique central to developing markers to assist breeders in molecular-based selection schemes. This

approach determines regions of the genome that contribute to complex, multi-genic traits, and allows the development of molecular probes that can be used to screen progeny in breeding populations for the desired allelic combinations of these genomic regions. QTLs for various traits in cacao have been identified, including resistance to fungal diseases, and various yield and morphological traits of interest such as fruit size and seed size (Lanaud et al. 1996; Motilal et al. 2000; Flament et al. 2001; Clement et al. 2003a, b; Queiroz et al. 2003; Risterucci et al. 2003). An example of the power of these methods to identify important traits is the QTL mapping of a major genomic locus conditioning resistance to the severe pathogen of cacao, *Crinipellis perniciosa* (Queiroz et al. 2003). In this study, an F2 population derived from a cross between the resistant Scavina-6 genotype and highly susceptible ICS-1 genotype was used. Mapping of 193 markers (AFLP and RAPD) resulted in the identification of several QTLs, including one major QTL responsible for 35% of the resistance. Using a related population of plants with an increased number of individuals (146 trees) and using co-dominant markers, Brown et al. (2005) created a linkage map using SSR markers and 12 candidate resistance genes. A much better genetic linkage map was produced using this approach, and two strong QTLs for resistance to witches' broom disease showing dominance were detected. In addition, a QTL for trunk diameter close to one of the resistance QTLs was found. Similar QTL maps have been produced for many different traits, as reviewed previously (Figueira and Alemanno 2005). Much of the currently available resources are now going into the generation of more markers, increasing marker density on maps and defining higher resolution to the major QTLs that have been identified for important disease resistances. Although not yet accomplished, it is likely that the coming years will see the first isolation of a cacao gene responsible for a QTL using a positional cloning approach, and the major QTL for resistance to witches' broom is a likely first target.

Schnell et al. (2005) published a study on the use of association genetic methods as applied to cacao. This method has been shown to be useful in human genetics and with other species where controlled populations and specific genetic designs are not possible. The application of this method to perennial tree crops could potentially circumvent some of the difficulties faced with the development of cacao populations for QTL mapping studies. In this study, microsatellite markers were used to identify loci associated with productivity in a selection of mature plants. The majority of these markers co-localized to QTLs previously identified for productivity by conventional QTL mapping (Clement et al. 2003a, b), demonstrating the utility of this approach. This method can potentially speed up the development of molecular markers useful for enhanced cacao breeding programs.

## 2.2 Gene Discovery

### 2.2.1 BAC Library Resources

Two cacao bacterial artificial chromosome (BAC) libraries have been constructed recently. A BAC library is useful for gene discovery in that it contains large fragments of genomic DNA each cloned into a bacterial strain that is arrayed in microtiter plates, making it simple to screen and to isolate specific genomic regions. Clement et al. (2004) reported on the construction of a BAC library from the genotype Scavina-6, which is one of the most well-known and utilized genotypes of cacao. This genotype was collected in 1938 near the Ucayali River in Peru on the eastern side of the Andes in the center of origin of cacao, the Amazon basin (Pound 1940). Seeds from a tree were used to produce a series of clones that still exist in Trinidad and from which cuttings have been disseminated around the world. Although poor yielding due to its small pods and seeds, the Scavina-6 genotype contains genes conditioning tolerance to several different fungal pathogens, and is used widely in breeding programs. This library contains approximately ten genome equivalents with an average insert size of 120 Kb. A second BAC library was created in collaboration between the USDA Miami Subtropical Horticultural Research Station and the Clemson BAC Resource Center, which also distributes this resource (<http://www.genome.clemson.edu>). This library was made using the genotype LCT-EEN 37 and represents approximately 11 genome equivalents with an average insert size of 120 Kb. These resources will facilitate genome mapping and gene discovery efforts in the future.

### 2.2.2 EST Resources for Cacao

Prior to 2002, only a handful of cacao DNA sequences were deposited in the NCBI GenBank database. Since then, the number has increased to the thousands. Expressed sequence tags (ESTs) are short stretches of DNA sequences determined from collections of cDNAs synthesized from RNA, and thus represent a snapshot of the genes expressed in a plant. Jones et al. (2002) published the first study using an EST approach to generate large numbers of expressed sequence tags for cacao. In this study, leaf and seed cDNA libraries were sequenced and a unigene set of 1380 sequences was assembled. Additionally, these sequences were used to create a microarray, which was used to demonstrate the specificity of tissue-specific expression of a number of genes. In a similar approach, Verica et al. (2004) used subtracted-normalized cDNA libraries to sequence a unigene set of 1256 gene fragments, some of which were shown to be up-regulated by a known inducer of the plant defense response Nep 1. The authors used suppressive-subtractive hybridization (SSH) and macroarray analysis to identify cacao ESTs representing genes induced by methyl jasmonate, ethylene, the salicylic acid analog benzothiadiazole (BTH)

and Nep1. A total of 475 clones that showed an increase of expression level of at least two-fold were designated as up-regulated clones and sequenced. Additionally, 1639 randomly chosen cDNAs were sequenced. After contig assembly (joining of overlapping sequences), 1256 unique non-overlapping sequences (unigenes) (367 contigs and 889 singletons) were obtained, including 330 representing up-regulated genes, and 865 unigenes were assigned to functional classes using BLAST, while 8% of the sequences up-regulated by the defense inducers were similar to defense proteins in other plants. The cDNAs isolated were similar to known defense-related genes from other plants, including heat shock proteins, NPR1 (a transcriptional regulator that mediates the expression of SA- and JA-responsive genes) and several PR genes, including chitinases, which have been shown to enhance resistance against fungal pathogens. An additional 8% of the up-regulated genes are predicted to play roles in signaling, although their roles in defense are unclear.

A major activity of INGENIC-MOL-BIOL is the establishment of a large EST database for cacao. This project, led by Claire Lanaud of CIRAD and implemented by the French CNS (Centre National de Séquençage), will sequence both ends of 100,000 clones from 28 cDNA libraries that have been contributed in a cooperative effort by the international cacao molecular biology community. All DNA sequencing data and clones will be made publicly available. The project is scheduled to be completed by mid 2007.

### 2.2.3 *Microarray Resources for Cacao*

A microarray is a specialized microscope slide on which thousands of small droplets of DNA are deposited, creating a substrate that can be used to assay the expression of thousands of genes simultaneously. In a major INGENIC-led collaboration, a unigene dataset of all known cacao DNA sequences including those described above was compiled in 2004 (Guiltinan, unpublished data). From 6659 sequences contributed, a 2781 sequence unigene set was assembled. From this set of sequences, a set of 50-mer oligonucleotides unique for each sequence was designed and synthesized and used to create a spotted microarray (Guiltinan, unpublished data). Examples of cacao genes represented on the microarray involved in plant defense response include basic endochitinase, phenylalanine ammonia-lyase, disease resistance protein (CC-NBS-LRR class), quercetin 3-O-methyltransferase 1/flavonol 3-O-methyltransferase 1/caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1), lipoxygenase (LOX2), pathogenesis-related protein (PR-1 protein), caffeoyl-CoA 3-O-methyltransferase, isoflavone reductase, chalcone synthase/naringenin-chalcone synthase, ethylene response sensor/ethylene-responsive sensor (ERS), catalase 1, endo-1,4- $\beta$ -glucanase, pathogen-responsive  $\alpha$ -dioxygenase and flavonol synthase 1 (FLS1). These microarrays are currently being evaluated for reproducibility and sensitivity in the author's laboratory and preliminary results are encouraging. In the near future, this array can be used to test the



effects of various conditions on cacao gene expression. For example, they can be used to ask questions such as, what genes are induced by endophytic microorganisms? Which genes are induced by pathogen infection and which genes are expressed differently in different genotypes?

#### 2.2.4 *Resistance Gene Analogs*

Plant disease resistance genes have been identified in a number of model plant species such as rice, maize, tomato and potato, and the results of these studies are beginning to be applied to cacao. Using a degenerate primer-PCR based approach, Lanaud et al. (2004) isolated a set of defense gene analogs from cacao, including several kinases similar to those shown in other species to be resistance genes, and several pathogenesis-related genes of the PR class 2 and 5 families. Interestingly, several of these genes appear to be clustered together, and reside on chromosomes in locations close to known QTLs for disease resistance. This is similar to results seen in other species, and may reflect an evolutionary process for the generation of multigene families for disease resistance. Ultimately, this approach could lead to map-based cloning of genes implicated in major QTLs for disease resistance.

Using a similar PCR-based strategy with degenerate primers based on highly conserved motifs within plant resistance genes, Kuhn et al. (2003) and Borrone et al. (2004) isolated a collection of cacao genomic fragments containing candidate resistance genes. One key class of pathogen resistance genes isolated, the so-called WRKY genes, are also found in many different plant species and have been shown to encode transcription factors involved in resistance to both biotic and abiotic stress. In both cases, multiple gene fragments were isolated and some of these were converted into polymorphic markers suitable for genetic mapping experiments, as discussed in Sects. 2.1.1 and 2.1.2. These types of markers are useful in that they encode potentially important genes for resistance, and thus are considered candidate genes. The sequences can also be used in detailed studies to understand the regulation of defense responses in cacao, and the evolution of plant resistance to pathogens.

#### 2.2.5 *Floral Development Genes*

Comparison of the developmental biology of cacao with *Arabidopsis* can provide insights into the conservation of the molecular mechanisms that determine cell fate, differentiation and signal transduction in cacao. One such system that has been studied is flower development, which is very well characterized in *Arabidopsis*. Research in the author's laboratory carried out by former graduate student J.D. Swanson addressed this comparison in a detailed study of cacao flower development from both the morphological and molecular levels of analysis (Swanson 2005). In addition to documenting the dynamics of cacao flower development, Swanson isolated a series of cacao floral-specific



genes using degenerate RT-PCR and used these to examine gene expression during early development using *in situ* hybridization. The results showed a remarkable conservation of expression pattern of these regulatory genes, each showing exquisite tissue and stage specificity, highlighting the high degree of conservation of the molecular mechanisms controlling flower development between these two related species. Major facts deriving from this research can be found on the Gultinan laboratory website (<http://gultinanlab.cas.psu.edu/research/cocoa/flowers.htm>).

### 2.3 Plant Tissue Culture and Cryopreservation

Somatic embryogenesis (SE) continues to be the primary method for *in vitro* propagation of cacao (Sondahl et al. 1993; Figueira and Janick 1995; Alemanno et al. 1997; Li et al. 1998; Traore 2000; Maximova et al. 2002). Although it has been applied to a wide variety of genotypes, a large variation in efficiency has been observed and this appears to be dependent on genotype. One factor, which has been attributed to the difficulty sometimes encountered in cacao tissue culture, is the high concentration of oxidized phenolic compounds that can accumulate. Alemanno et al. (2003) published a study that investigated the phenolic composition of cocoa floral explants and how they change during culture. Using this knowledge and the methods developed, it may be possible to modify the phenolic composition or change the oxidation status of cacao tissue cultures and thus improve the efficiency and/or genotype variation of cacao embryogenesis. In a more recent study of cacao somatic embryogenesis, Santos et al. (2005) investigated the somatic embryogenesis receptor kinase (SERK) of cacao, which is known to be highly expressed and essential during SE in other plants. These authors found that the cacao genome contains a single functional copy of the SERK gene and that this gene is expressed during SE. Since SERK gene expression is required for SE to proceed in other species, this raises the interesting possibility of using the SERK gene as a marker for SE in cacao, and as a predictor of the potential for SE capacity for different genotypes, although this idea has yet to be tested. A discussion of the use of molecular markers to investigate genetic changes that might occur during SE in cacao (Rodriguez Lopez et al. 2004) is given in Section 2.1.1.

Cryopreservation of cacao has been long viewed as a promising application of biotechnology to cacao conservation, considering the loss of wild cacao germplasm due to deforestation, and the risk of loss of germplasm stored in living collections. After development of SE systems in the mid to late 1990s, cryopreservation systems were developed for cacao (Florin et al. 2000; Fang et al. 2004). In the most recent of these publications, Fang et al. (2004) describes a cryopreservation method using encapsulation and dehydration. Absciscic acid (ABA) and sugars were used as cryoprotectants during a preculture step. Three genotypes were preserved with a 33% recovery rate, and recovered plants were phenotypically identical to non-cryopreserved SE-derived plants.

Work performed at the Nestlé's research center in Tours, France, has developed a cryopreservation system based on a different approach (Florin et al. 2000; V. Pétiard/B. Florin, pers. comm.). This system makes use of hormonally induced floral explants (staminodes) that are then pretreated and frozen prior to SE development. After thawing, the induced staminodes recover and proceed directly to SE, with excellent recovery rates. This process has the advantage of requiring less time prior to freezing and this would be a big advantage for large-scale germplasm preservation projects.

## 2.4 Haploid Plants

Doubled haploids have long been considered as an important potential method to generate homozygous lines of cacao for the production of hybrid seed for germplasm propagation (Dublin 1973; Lanaud 1987a, b, 1988a, b; Sounigo et al. 2003). However, progress in this area has been slow. A publication by Sounigo et al. (2003) documented the work carried out in this area and summarized the conclusions to date. In this study, twelve doubled haploids were used as parents in field trials in the Ivory Coast, West Africa. The results showed that some of the doubled haploids showed a significantly higher combining value than their parents, demonstrating the potential of this technology for rapid improvement of parents. The authors concluded that many more crosses between doubled haploids would need to be tested to identify potential crosses for improving cacao germplasm. This is a challenging but potentially very rewarding proposition that could lead to the development of seed production systems for cacao germplasm distribution and plantation improvement. Notably, tissue culture has yet to be applied to the development of doubled haploids of cacao.

## 2.5 Genetic Transformation

Genetic transformation offers a tool for performing basic research on gene structure and function, and also a means to potentially introduce genes from other organisms for crop improvement. In cacao, an initial report demonstrated the susceptibility of cacao cells to *Agrobacterium*, a commonly used bacterium capable of introducing DNA into plant cells (Purdy and Dickstein 1989), and another manuscript described the transformation of cacao cells (Sain et al. 1994). Two reports of using particle bombardment (the use of high velocity gold particles to introduce DNA into cultured plant cells) have been reported (Perry et al. 2000; Santos et al. 2002). In both of these studies, the particle bombardment method was used to demonstrate that reporter genes could be introduced into cacao cells and visualized. The method was further refined through optimization of a pretreatment step with osmotic adjustment that increased transient transformation frequencies. However, none of these efforts resulted in the regeneration of transgenic cacao plants. While regeneration of cacao plants through somatic embryogenesis was possible, as was the

introduction of DNA into cacao tissue cultured cells, the ability to regenerate whole plants from the individual transformed cells and cell clusters remained elusive.

Using *A. tumefaciens*-based transformation of cultured somatic embryos, a transformation system for cacao capable of producing whole plants was established recently in this laboratory (Maximova et al. 2003). Using the green fluorescent protein marker gene as a way to identify transgenic somatic embryos, the authors recovered a series of transgenic plants that were grown to maturity. Growth and development of the plants was shown to be the same as that of the control, untransformed plants. Transgene insertion, gene expression and stability were all shown to be similar to that in other transgenic plants of different species. Details of some of the experiments in which various parameters were optimized were also published separately (Traore 2000; Antunez de Mayolo et al. 2003).

Cacao plants were grown to maturity and the transgenes were shown to be stable through seed to the next generation (Guiltinan, unpublished data). More recently, this system has been used to demonstrate the function of a cacao chitinase gene in plant defense against fungal pathogens (Maximova et al., 2006).

This has shown that the cacao transformation system can be useful in analysis of gene function. The utility of this system for crop improvement, however, remains to be seen, as continued opposition by the public to genetically manipulated crops continues to be debated. Because of this controversy, there are currently no experiments in progress or planned by the author involving a field release of these plants in any country.

## 2.6 Cacao Pests and Pathogens

### 2.6.1 Plant-Pathogen Interactions

Researchers are beginning to use newly discovered cacao genes as probes to investigate various aspects of cacao development and, in particular, their responses to interactions with pathogenic organisms. Bailey et al. (2005a,b) recently published two manuscripts in which the expression of genes in cacao leaves under different stress or induction conditions was evaluated. These studies have revealed interactions between gene expression changes during leaf development and in response to various inducers such as ethylene, wounding, methyl jasmonate and pathogen infection. This is a starting point from which to begin dissecting these pathways for a better understanding of the metabolic changes related to resistance to pathogens in cacao and the underlying differences between cacao genotypes. Such knowledge will provide a fundamental background of resistance mechanisms that will be helpful to assist the planning of breeding programs in the future.

Using a physiological and metabolic approach, Aneja and Gianfagna (2001) demonstrated a large increase in caffeine levels in stems and leaves in response

to wounding, pathogen infection and by treatment with salicylic acid and benzoethiadiazole, inducers of the plant defense responses. Caffeine was shown to inhibit the growth of *Crinipellis perniciosus* in vitro. This finding is consistent with the later finding that the gene encoding caffeine synthase is induced in cacao leaves in response to various defense response inducers (Bailey et al. 2005a, b). In a more comprehensive metabolic study, Scarpari et al. (2005) investigated changes in a large number of metabolic compounds during the infection and development of witches' broom disease. Changes in sugars, amino acids, alkaloids, tannins, chlorophyll, fatty acids, glycerol carotenoids, xanthophylls and notably ethylene were seen during the infection process. Since ethylene is a well known plant hormone, the authors suggest that it may play a key role in the formation of brooms during disease development. This study sets a new precedence for the comprehensive analysis of metabolites in the investigation of cacao-pathogen interactions.

### 2.6.2 Associations with Beneficial Microorganisms and Biocontrol

Endophytic and epiphytic fungi and bacteria have been shown to live on the surfaces and inside of most plant species studied to date and, in cacao, this has been intensely studied in the last few years because of the potential beneficial use of these organisms as biocontrol agents for protection against cacao pathogens. A recent study characterized some of the naturally occurring endophytic fungi in leaves of cacao growing in Panama (Arnold et al. 2003). A wide range of fungal species was found in cacao leaves; up to 13 different taxa in a single leaf sample and 344 different morphotaxa were identified overall. The authors also showed that the presence of the endophytes significantly decreased leaf necrosis and mortality when plants were challenged with *Phytophthora* sp., and suggested that this protection may be due to changes in leaf chemistry, a hypothesis consistent with the observations described above with caffeine level changes in cacao leaves.

There are several devastating insect pests of cacao, the worst of which include the myriids of West Africa and the cocoa pod borer (CPB) of Indonesia. In an effort to find potential insecticidal proteins active against CPB, Santoso et al. (2004) screened 12 Cry proteins from *Bacillus thuringiensis* for activity against CPB larvae. Five of the toxins were shown to be more active, opening up the possibility of using these proteins or the genes encoding them as insecticides.

### 2.6.3 Molecular Analysis of Pathogen Diversity

Molecular biology and biotechnology is also being increasingly applied in attempts to understand more about the genetic structure of cacao pests and diseases, and to find ways to fight them. For example, PCR and DNA sequencing have been applied to the analysis of the genetic variability of the cocoa swollen shoot virus, a major pathogen in parts of West Africa (Muller et al. 2001;

Muller and Sackey 2005). In the most recent of these studies, the genomes of five isolates were sequenced and compared to the reference sequence. Up to 29.4% sequence variability was detected and these differences could be related to the geographic distributions of the isolates. Interestingly, one of the proposed open reading frames of the CSSV genome, ORF X, varied greatly in size between several of the isolates. The authors suggest that this may indicate that this open reading frame may actually be non-functional.

Using similar approaches, researchers have studied the genetic structures and evolutionary relatedness of a number of cacao fungal pathogens (Niella et al. 2000; Appiah et al. 2003; Chowdappa et al. 2003; de Arruda et al. 2003a, b; Evans et al. 2003; Ploetz et al. 2005). These studies are beginning to shed light on the evolution, geographic distribution and movements of the important cacao fungal pathogens. In an analysis of the fungal pathogen responsible for frosty pod rot (*Moniliophthora roreri*), Phillips (2003) demonstrated the very close relationship of this species to another cacao pathogen, *Crinipellis perniciosa*. Phylogenetic analysis of ribosomal RNA sequences from both nuclear and mitochondrial genes were compared with sequences from other fungi, clearly supporting the re-assignment of *M. roreri* into the Basidiomycete and its close relatedness to *C. perniciosa*. The rapidly advancing understanding of relationships between cacao pathogens and their population structures will contribute to accelerated plant breeding for durable, horizontal resistance.

### 3 Future Prospects

#### 3.1 Translational Genomics

*Theobroma cacao* is a simple diploid with ten chromosomes ( $2n = 2x = 20$ ) and, for plant species, a small genome. Published genome size estimates vary from 390 to 415 Mb (Figueira et al. 1992; Couch et al. 1993). *Theobroma cacao* is a member of the order Malvales, which includes the important crop plant cotton. Both are members of the Eurosids II group of plants that contains the Brassicales, including *Arabidopsis* (Soltis et al. 2002). The close evolutionary relatedness of these three species suggests that cotton and cacao are excellent crop plants for translational research. What can be learned from the model plant species cotton and *Arabidopsis* and how can this be used to speed up cacao improvement? These questions are a central focus of the author's current research objectives. The recent advances in methodologies and strategies for whole genome sequencing, and the dramatic reduction in costs, makes it highly likely that the cacao genome will be completely sequenced in the next 5 years. With the full genome sequence, translational genomics will be greatly accelerated, and cacao molecular biologists would be relieved of tedious marker development and gene discovery efforts, and be able to focus more directly on gene function and trait mapping for crop improvement. Bioin-

formatics will become increasingly important to the future of cacao genetics research.

### 3.2 Marker-Assisted Selection-Based Breeding

While the accomplishments reviewed here represent an encouraging beginning towards the applications of genomics to cacao breeding, in order to realize the practical benefits of marker-assisted selection, a much more extensive set of genomics resources is needed. In the future, with such resources available, it is envisaged that plant breeders will use marker-assisted selection to vastly speed up cacao breeding programs. In addition to genetic markers, metabolic markers, such as caffeine concentrations, could be used for breeding purposes, once the specific mechanisms important to disease resistance are better understood. In the future, high-throughput metabolic analysis could be used for screening germplasm collections and progeny of breeding trials.

In the near future, local accessions, well adapted to regional environmental and soil conditions, will be crossed with internationally tested genotypes with disease resistance, high yield and other quality traits. Molecular markers will be used to screen segregating progeny for desired traits while retaining locally desired adaptive phenotypes. Preemptive breeding for resistance to diseases not yet spread to local areas can be included in selection schemes. Flavor and other quality traits will also need to be maintained or improved simultaneously. Gene pyramiding will be used to enhance resistance durability. Selected progenies will be vegetatively propagated through a combination of tissue culture, grafting and rooted cuttings, for distribution to farmers. The genetic diversity of the wild cacao germplasm will be safeguarded in large cryopreservation storehouses.

### 3.3 Increased Scientific Capacity

To reach these goals, and to have a lasting impact, it is also essential that we increase the scientific capacity of cocoa-producing countries. This can be accomplished through graduate and postgraduate education, exchange visits and training workshops, community building and user-oriented bioinformatics resources. An important complementary component will be the training of scientists in the developed countries in international agriculture, in addition to their training in plant molecular genetics, genomics and bioinformatics, to form a future cadre of well-educated plant scientists versed and experienced in international agricultural issues. While cocoa does not fight hunger directly as it is an export crop, it is an important cash crop for farmers who otherwise can grow most of their own food for sustenance, but need money to improve their quality of life or to purchase food they cannot grow. Our investments now in research and education in the basic plant sciences, genetics and molecular biology of cacao will help to ensure the well-being of the cacao farmer in the future.

**Acknowledgements.** The author would like to thank the many colleagues who responded to requests for reprints and suggestions for articles which may otherwise have been overlooked, and Drs. Jose R. Peralta-Videa, Antonio Figueira, Lizz Johnson and Basil Bartley for critical reading and comments on the text.

## References

- Alemanno L, Berthouly M, Michaux-Ferriere N (1997) A comparison between *Theobroma cacao* L. zygotic embryogenesis and somatic embryogenesis from floral explants. *In Vitro Cell Dev Biol-Plant* 33:163–172
- Alemanno L, Ramos T, Gargadenec A, Andary C, Ferriere N (2003) Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. *Ann Bot* 92:613–623
- Alverson WS, Whitlock BA, Nyffeler R, Bayer C, Baum DA (1999) Phylogeny of the core Malvales: evidence from NDHF sequence data. *Am J Bot* 86:1474–1486
- Aneja M, Gianfagna T (2001) Induction and accumulation of caffeine in young, actively growing leaves of cocoa (*Theobroma cacao* L.) by wounding or infection with *Crinipellis pernicios*a. *Physiol Mol Plant Pathol* 59:13–16
- Antunez de Mayolo G, Maximova SN, Pishak S, Guiltinan MJ (2003) Moxalactam as a counter-selection antibiotic for *Agrobacterium*-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis. *Plant Sci* 164:607–615
- Appiah AA, Flood J, Bridge PD, Archer SA (2003) Inter- and intraspecific morphometric variation and characterization of *Phytophthora* isolates from cocoa. *Plant Pathol* 52:168–180
- Appiah AA, Flood J, Archer SA, Bridge PD (2004) Molecular analysis of the major *Phytophthora* species on cocoa. *Plant Pathol* 53:209–219
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci USA* 100:15649–15654
- Atkinson MD, Withers LA, Simpson MJA (1986) Characterization of cacao germplasm using isoenzyme markers. 1. A preliminary survey of diversity using starch gel electrophoresis and standardisation of the procedure. *Euphytica* 35:741–750
- Bailey B, Bae H, Strem MD, Antunez de Mayolo G, Guiltinan MJ (2005a) Gene expression in leaves of *Theobroma cacao* in response to mechanical wounding, ethylene, or methyl jasmonate. *Plant Sci* 128:1247–1258
- Bailey B, Bae H, Strem MD, Mayolo GA, Guiltinan M, Verica JA, Maximova S, Bowers JH (2005b) Developmental expression of stress response genes in *Theobroma cacao* leaves and their response to Nep1 treatment and a compatible infection by *Phytophthora megakarya*. *Plant Physiol Biochem* 43:611–622
- Bartley BGD (2005) The genetic diversity of cacao and its utilization. CAB International, Wallingford
- Bayer C, Fay ME, De Bruijn AY, Savolainen V, Morton CM, Kubitzki K, Alverson WS, Chase MW (1999) Support for an expanded family concept of Malvaceae within a circumscribed order Malvales: a combined analysis of plastid atpB and rbcL DNA sequences. *Bot J Linn Soc* 129:267–303
- Bennett AB (2003) Out of the Amazon: *Theobroma cacao* enters the genomic era. *Trends Plant Sci* 8:561–563
- Borrone JW, Kuhn DN, Schnell RJ (2004) Isolation, characterization, and development of WRKY genes as useful genetic markers in *Theobroma cacao*. *Theor Appl Genet* 109:495–507
- Bowers JH, Bailey BA, Hebbar PK, Sanogo S, Lumsden RD (2001) The impact of plant diseases on worldwide chocolate production. <http://www.plantmanagementnetwork.org/pub/php/review/cacao/>



- Brown JS, Kuhn DN, Lopez U, Schnell RJ (2005) Resistance gene mapping for witches' broom disease in *Theobroma cacao* L. in an F2 population using SSR markers and candidate genes. *J Am Soc Hortic Sci* 130:366–373
- Charters YM, Wilkinson MJ (2000) The use of self-pollinated progenies as “in-groups” for the genetic characterization of cocoa germplasm. *Theor Appl Genet* 100:160–166
- Chowdappa P, Brayford D, Smith J, Flood J (2003) Molecular discrimination of *Phytophthora* isolates on cocoa and their relationship with coconut, black pepper and bell pepper isolates based on rDNA repeat and AFLP fingerprints. *Curr Sci* 84:1235–1237
- Clement D, Risterucci AM, Motamayor JC, N’Goran J, Lanaud C (2003a) Mapping QTL for yield components, vigor, and resistance to *Phytophthora palmivora* in *Theobroma cacao* L. *Genome* 46:204–212
- Clement D, Risterucci AM, Motamayor JC, Ngoran J, Lanaud C (2003b) Mapping quantitative trait loci for bean traits and ovule number in *Theobroma cacao* L. *Genome* 46:103–111
- Clement D, Lanaud C, Sabau X, Fouet O, Le Cunff L, Ruiz E, Risterucci AM, Glaszmann JC, Piffanelli P (2004) Creation of BAC genomic resources for cocoa (*Theobroma cacao* L.) for physical mapping of RGA containing BAC clones. *Theor Appl Genet* 108:1627–1634
- Coe SD, Coe MD (1996) The true history of chocolate. Thames and Hudson, New York
- Couch JA, Zintel HA, Fritz PJ (1993) The genome of the tropical tree *Theobroma cacao* L. *Mol Gen Genet* 237:123–128
- Crouzillat D, Phillips W, Fritz PJ, Petiard V (2000) Quantitative trait loci analysis in *Theobroma cacao* using molecular markers. Inheritance of polygenic resistance to *Phytophthora palmivora* in two related cacao populations. *Euphytica* 114:25–36
- Cruz M, Whitkus R, Gomez-Pompa A, Monta-Bravo L (1995) Origins of cacao cultivation. *Nature* 375:542–543
- Cryer NC, Fenn MGE, Turnbull CJ, Wilkinson MJ (2006) Allelic size standards and reference genotypes to unify international cocoa (*Theobroma cacao* L.) microsatellite data. *Genetic Resour Crop Evol*, <http://dx.doi.org/10.1007/s10722-005-1286-9>
- Day RK (1984) Population dynamics of cocoa pod borer *Acrocerops cramerella*: importance of host plant cropping cycle. In: *Proc Int Conf on Cocoa and Coconuts*, Kuala Lumpur, pp 1–9
- de Arruda MC, Ferreira MA, Miller RN, Resende ML, Felipe MS (2003a) Nuclear and mitochondrial rDNA variability in *Crinipellis perniciosa* from different geographic origins and hosts. *Mycol Res* 107:25–37
- de Arruda MC, Miller RN, Ferreira MASV, Felipe MS (2003b) Comparison of *Crinipellis perniciosa* isolates from Brazil by ERIC repetitive element sequence-based PCR genomic fingerprinting. *Plant Pathol* 52:236–244
- Dias LAS (2001) Genetic improvement of cacao. <http://ecoport.org/ep?searchtype=earticleview&earticleid=197>
- Dublin MP (1973) Phytogénétique – haploïdie chez *Theobroma cacao*. *C R Acad Sci Fr* 276:757–759
- Efombagn MIB, Marelli JP, Ducamp M, Cilas C, Nyasse S, Vefonge D (2004) Effects of fruiting traits on the field resistance of cocoa (*Theobroma cacao* L.) clones to *Phytophthora megakarya*. *Phytopathology* 152:557–562
- Emch M (2003) The human ecology of Mayan cacao farming in Belize. *Human Ecol* 31:111–132
- Evans HC, Holmes KA, Reid AP (2003) Phylogeny of the frosty pod rot pathogen of cocoa. *Plant Pathol* 52:476–485
- Faleiro FG, Pires JL, Monteiro WR, Lopes UV, Yamada MM, Piedra AG, Moura AD, Arevalo-Gardini EA, Marques J, Gramacho K, Faleiro A, Santos M (2004) Variability in cacao accessions from the Brazilian, Ecuadorian, and Peruvian Amazons based on molecular markers. *Crop Breed Appl Biotechnol* 4:227–233
- Fang JY, Wetten A, Hadley P (2004) Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. *Plant Sci* 166:669–675
- Figueira A, Alemanno L (2005) *Theobroma cacao* (cacao). In: Litz RE (ed) *Biotechnology of fruit and nut crops*. CAB International Biosciences, Wallingford, Oxon, pp 639–669
- Figueira A, Janick J (1995) Somatic embryogenesis in cacao (*Theobroma cacao* L.). In: Newton R (ed) *Somatic embryogenesis in woody plants*, vol 2. Kluwer, Dordrecht, pp 291–310

- Figueira A, Janick J, Goldsbrough P (1992) Genome size and DNA polymorphism in *Theobroma cacao*. J Am Hortic Sci 117:673–677
- Figueira A, Janick J, Levy M, Goldsbrough P (1994) Reexamining the classification of *Theobroma cacao* L. using molecular markers. J Am Hortic Sci 119:1073–1082
- Flament MH, Kebe I, Clement D, Pieretti I, Risterucci AM, N’Goran JA, Cilas C, Despreaux D, Lanaud C (2001) Genetic mapping of resistance factors to *Phytophthora palmivora* in cocoa. Genome 44:79–85
- Florin B, Brulard E, Pétiard V (2000) In vitro cryopreservation of cacao genetic resources. In: Engelman F, Tagaki H (eds) Cryopreservation of tropical plant germplasm. Japanese International Research Centre for Agricultural Sciences and IPGRI, Rome, pp 344–347
- Fritz PJ, Osei JK, Goodin MM, Furtek DB (1991) Molecular and biochemical markers for genetic analysis of *Theobroma cacao*. In: Proc Int Cocoa Res Conf, Kuala Lumpur, pp 391–400
- Gomes LMC, Melo GRP, Faleiro FG, Silva SDM, Araujo RC, Bahia RC, Morales MG, Ahnert D (2000) Genetic diversity of *Crinipellis pernicioso* from the south region of Bahia-Brazil using RAPD molecular markers. Fitopatologia Brasil 25:377
- Guyton B, Lumsden R, Matlick BK (2003) Strategic plan for sustainable cocoa production. Manufact Confect 83:55–60
- Hanna AD (1954) Application of a systemic insecticide by trunk implantation to control a mealy-bug vector of the cacao swollen shoot virus. Nature 173:730–731
- Hervé L, Djiekpor E, Jacquemond M (1991) Characterization of the genome of cacao swollen shoot virus. J Gen Virol 72:1735–1739
- Iwano AD, Bekele FL, Butler DR (2003) Evaluation and utilisation of cacao (*Theobroma cacao* L.) germplasm at the International Cocoa Genebank, Trinidad. Euphytica 130:207–221
- Johnson L (2003) INGENIC Workshop, cocoa genomics group. Gro Cocoa 4:4–5 (<http://www.cabicommodities.org/acc/acrc/pdffiles/groc/groc.htm>)
- Jones PG, Allaway D, Gilmour DM, Harris C, Rankin D, Retzel ER, Jones CA (2002) Gene discovery and microarray analysis of cacao (*Theobroma cacao* L.) varieties. Planta 216:255–264
- Keane PJ (1992) Diseases and pests of cocoa: an overview. Cocoa pest and disease management in Southeast Asia and Australasia. FAO Plant Prod Prot Pap 112:1–12
- Kuhn DN, Schnell RJ (2005) Use of capillary array electrophoresis single strand conformational polymorphism analysis to estimate genetic diversity of candidate genes in germplasm collections. Meth Enzymol 395:238–258
- Kuhn DN, Heath M, Wissner RJ, Meerow A, Brown JS, Lopes U, Schnell RJ (2003) Resistance gene homologues in *Theobroma cacao* as useful genetic markers. Theor Appl Genet 107:191–202
- Kuhn DN, Borrone J, Meerow AW, Motamayor JC, Brown JS, Schnell RJ (2005) Single-strand conformation polymorphism analysis of candidate genes for reliable identification of alleles by capillary array electrophoresis. Electrophoresis 26:112–125
- Lanaud C (1987a) Doubled haploids of cocoa (*Theobroma cacao* L.). I. Observations of fertility. Plant Breed 99:187–195
- Lanaud C (1987b) Doubled haploids of cocoa (*Theobroma cacao* L.). II. Observations of monogenic and polygenic characters. Plant Breed 99:196–202
- Lanaud C (1988a) Behavior in crossing of the haploids of cacao-trees (*Theobroma cacao*). Can J Bot 66:1986–1992
- Lanaud C (1988b) Origin of haploids and semigamy in *Theobroma cacao* L. Euphytica 38:221–228
- Lanaud C, Kebe I, Risterucci AM, Clement D, N’Goran JKA, Grivet L, Tahi M, Cilas C, Pieretti I, Eskes AB, Despreaux D (1996) Mapping quantitative trait loci (QTL) for resistance to *Phytophthora palmivora* in *T. cacao*. In: Proc 12th Int Cocoa Res Conf, Salvador, Bahia, pp 99–105
- Lanaud C, Risterucci AM, Pieretti I, Falque M, Bouet A, Lagoda PJ (1999) Isolation and characterization of microsatellites in *Theobroma cacao* L. Mol Ecol 8:2141–2152
- Lanaud C, Risterucci AM, Pieretti I, N’goran JAK, Fargeas D (2004) Characterisation and genetic mapping of resistance and defence gene analogs in cocoa (*Theobroma cacao* L.). Mol Breed 13:211–227

- Lerceteau E, Robert T, Petiard V, Crouzillat D (1997) Evaluation of the extent of genetic variability among *Theobroma cacao* accessions using RAPD and RFLP markers. *Theor Appl Genet* 95:10–19
- Li Z, Traore A, Maximova S, Guiltinan MJ (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. *In Vitro Cell Dev Biol-Plant* 34:293–299
- Marita JM, Nienhuuis J, Pires JL, Aitken WM (2001) Analysis of genetic diversity in *Theobroma cacao* with emphasis on witches' broom disease resistance. *Crop Sci* 41:1305–1316
- Maximova SN, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan MJ (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In Vitro Cell Dev Biol-Plant* 38:252–259
- Maximova SN, Miller C, Antunez de Mayolo G, Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. *Plant Cell Rep* 21:872–883
- Maximova SN, Marelli J-P, Young A, Pishak S, Verica JA, Guiltinan MJ (2006) Over-expression of a cacao class I chitinase gene in *Theobroma cacao* L. enhances resistance against the pathogen, *Colletotrichum gloeosporioides*. *Planta* 224(4):740–749
- Morais R (2005) The Gnomes of cocoa. *Forbes Magazine*, New York, 14 March, pp 110–112
- Motamayor JC, Risterucci AM, Lopez PA, Ortiz CF, Moreno A, Lanaud C (2002) Cacao domestication I: the origin of the cacao cultivated by the Mayas. *Heredity* 89:380–386
- Motamayor JC, Risterucci AM, Heath M, Lanaud C (2003) Cacao domestication II: progenitor germplasm of the Trinitario cacao cultivar. *Heredity* 91:322–330
- Motilal LA, Sounigo O, Thevenin JM, Howell MH, Pieretti I, Risterucci AM, Noyer JL, Lanaud C (2000) *Theobroma cacao* L.: genome map and QTLs for *Phytophthora palmivora* resistance. In: *Proc 13th Annu Cocoa Res Conf*, Kota Kinabalu
- Muller E, Sackey S (2005) Molecular variability analysis of five new complete cacao swollen shoot virus genomic sequences. *Arch Virol* 150:53–66
- Muller E, Jacquot E, Yot P (2001) Early detection of cacao swollen shoot virus using the polymerase chain reaction. *J Virol Meth* 93:15–22
- Niella GR, Resende MLV, De Castro HA, Figueira AR, Silva SDM, Araujo IS, Gomes LMC, Faleiro FG (2000) Genetic diversity of the monosporic isolates of *Crinipellis perniciosus* from different Brazilian states, using RAPD markers. *Fitopatol Brasil* 25:400
- Nyasse S, Despreaux D, Cilas C (2002) Validity of a leaf inoculation test to assess the resistance to *Phytophthora megakarya* in a cocoa (*Theobroma cacao* L.) diallel mating design. *Euphytica* 123:395–399
- Opoku IY, Akrofi AY, Appiah AA (2002) Shade trees are alternative hosts of the cocoa pathogen *Phytophthora megakarya*. *Crop Prot* 21:629–634
- Pereira JL, Ram A, Defigueiredo JM, Dealmeida LCC (1990) First occurrence of witches' broom disease in the principal cocoa-growing region of Brazil. *Trop Agric* 67:188–189
- Perry M, Power J, Lowe K, MR D (2000) Biolistic transformation of cacao (*Theobroma cacao* L.). *Trop Agric* 77:64–66
- Phillips W (2003) Origin, biogeography, genetic diversity and taxonomic affinities of the cacao fungus *Moniliophthora roreri* as determined using molecular, phytopathological and morpho-physiological evidence. PhD thesis, University of Reading .
- Piasentin F, Klare-Repnik L (2004) Biodiversity conservation and cocoa agroforests. *Gro Cocoa* 5:7–8 (<http://www.cabi-commodities.org/acc/accrc/pdf/files/groc/groc.htm>)
- Ploetz RC, Schnell RJ, Ying Z, Zheng Q, Olano CT, Johnson E, Motamayor JC (2005) Molecular diversity in *Crinipellis perniciosus* with AFLPs. *Eur J Plant Pathol* 111:317–326
- Pound FJ (1940) Witches' broom resistance in cacao. *Trop Agric* 17:6–8
- Pugh T, Fouet O, Risterucci AM, Brottier P, Abouladze M, Deletrez C, Courtois B, Clement D, Larmande P, Ngoran JAK (2004) A new cacao linkage map based on codominant markers: development and integration of 201 new microsatellite markers. *Theor Appl Genet* 108:1151–1161

- Purdy LH, Dickstein ER (1989) *Theobroma cacao*, a host for *Agrobacterium tumefaciens*. Plant Dis 73:638–639
- Queiroz VT, Guimaraes CT, Anheret D, Schuster I, Daher RT, Pereira MG, Miranda VRM, Loguericio LL, Barros EG, Moreira MA (2003) Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches' broom disease. Plant Breed 122:268–272
- Rice RA, Greenberg R (2003) The chocolate tree: growing cacao in the forest. Nat His 112:36–43
- Risterucci AM, Grivet L, Ngoran JA, Pieretti I, Flament MH, Lanaud C (2000) A high-density linkage map of *Theobroma cacao* L. Theor Appl Genet 101:948–955
- Risterucci AM, Paulin D, Ducamp M, N'Goran JAK, Lanaud C (2003) Identification of QTLs related to cocoa resistance to three species of *Phytophthora*. Theor Appl Genet 108:168–174
- Rodriguez Lopez CM, Wetten AC, Wilkinson MJ (2004) Detection and quantification of in vitro-culture induced chimerism using simple sequence repeat (SSR) analysis in *Theobroma cacao* (L.). Theor Appl Genet 110:157–166
- Ronning CM, Schnell RJ, Kuhn DN (1995) Inheritance of random amplified polymorphic DNA (RAPD) markers in *Theobroma cacao* L. J Am Soc Hortic Sci 120:681–686
- Ruf F, Zadi H (2003) Cocoa: from deforestation to reforestation. <http://nationalzoo.si.edu/conservationandscience/migratorybirds/research/cacao/ruf.cfm>
- Sain SL, Oduro KK, Furtak DB (1994) Genetic transformation of cocoa leaf cells using *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 37:342–351
- Santos M, Albuquerque de Barros E, Tinoco M, Brasileiro A, Aragão F (2002) Repetitive somatic embryogenesis in cacao and optimisation of gene expression by particle bombardment. J Plant Biotechnol 4:71–76
- Santos MO, Romano E, Yotoko K, Tinoco M, Dias B, Aragao F (2005) Characterisation of the cacao somatic embryogenesis receptor-like kinase (SERK) gene expressed during somatic embryogenesis. Plant Sci 168:723–729
- Santoso D, Chaidamsari T, Wiryadiputra S, de Maagd RA (2004) Activity of *Bacillus thuringiensis* toxins against cocoa pod borer larvae. Pest Manag Sci 60:735–738
- Saunders JA, Mischke S, Leamy EA, Hemeida AA (2004) Selection of international molecular standards for DNA fingerprinting of *Theobroma cacao*. Theor Appl Genet 110:41–47
- Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AWV, Schiavinato MA, Cascardo JCM, Pereira GAG (2005) Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis pernicioso*. J Exp Bot 56:865–877
- Schnell RJ, Olano CT, Brown JS, Meerow AW, Cervantes-Martinez C, Nagai C, Motamayor JC (2005) Retrospective determination of the parental population of superior cacao (*Theobroma cacao* L.) seedlings and association of microsatellite alleles with productivity. J Am Soc Hortic Sci 130:181–190
- Sereno M, Albuquerque P, Vencovsky R, Figueira A (2006) Genetic diversity and natural population structure of cacao (*Theobroma cacao* L.) from the Brazilian Amazon evaluated by microsatellite markers. Conser Genet 7(1):13–24
- Soltis DE, Soltis PS, Albert VA, Oppenheimer DG, DePamphilis CW, Ma H, Frohlich MW, Theissen G (2002) Missing links: the genetic architecture of flower and floral diversification. Trends Plant Sci 7:22–31
- Sondahl MR, Liu S, Bellato C, Bragin A (1993) Cacao somatic embryogenesis. Acta Hort 336:245–248
- Sounigo O, Lachenaud P, Bastide P, Cilas C, N'Goran J, Lanaud C (2003) Assessment of the value of doubled haploids as progenitors in cocoa (*Theobroma cacao* L.) breeding. J Appl Genet 44:339–353
- Sousa Silva CRS, Figueira A (2005) Phylogenetic analysis of *Theobroma* (Sterculiaceae) based on Kunitz-like trypsin inhibitor sequences. Plant Syst Evol 250:93–104
- Swanson JD (2005) Flower development in *Theobroma cacao* L.: an assessment of morphological and molecular conservation of floral development between *Arabidopsis thaliana* and *Theobroma cacao*. PhD Diss, Pennsylvania State University

- Traore A (2000) Somatic embryogenesis, embryo conversion, micropropagation and factors affecting genetic transformation of *Theobroma cacao* L. PhD thesis, Pennsylvania State University
- Verica JA, Maximova SN, Strem MD, Carlson JE, Bailey BA, Guiltinan MJ (2004) Isolation of ESTs from cacao (*Theobroma cacao* L.) leaves treated with inducers of the defense response. *Plant Cell Rep* 23:404–413
- Whitkus R, Delacruz M, Motabravo L, Gomezpompa A (1998) Genetic diversity and relationships of cacao (*Theobroma cacao* L.) in southern Mexico. *Theor Appl Genet* 96:621–627
- Whitlock BA, Bayer C, Baum DA (2001) Phylogenetic relationships and floral evolution of the Byttnerioideae (“Sterculiaceae” or Malvaceae s.l.) based on sequences of the chloroplast gene, *ndhF*. *Syst Bot* 26:420–437
- Wilde J, Waugh R, Powell W (1991) Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. *Theor Appl Genet* 83:871–877
- Wood GAR, Lass RA (1985) *Cocoa*, 4th edn. Longman Scientific and Technical, New York

### III.3 Tea

T.K. MONDAL<sup>1</sup>

#### 1 Introduction

Tea serves as the most important morning drink for two-thirds of the world populace because of its attractive aroma and taste. It has become one of the most important agro-based, ecofriendly, labour-intensive, employment-generating, export-oriented industries in the developing countries. India ranks as the most important producer, consumer and exporter of black tea in the world. Tea originated in southeast Asia, specifically around the intersection of latitude 29°N and longitude 98°E, the point of confluence of the lands of northeast India, north Burma, southwest China and Tibet. The plant was introduced to more than 52 countries, from this 'centre of origin'.

Tea (*Camellia sinensis* L.) belongs to the family Theaceae, and a woody, cross-pollinated, perennial plantation crop that can grow naturally up to 15 m in height. However, under cultivation condition, a bush height of 60–100 cm is maintained for harvesting the young leaves. The flowers are white in colour and born singly or in pairs at the leaf axils. The fruits are green in colour with two to three seeds and are borne within 5–6 years after planting of the bushes. Leaf size is the main criterion used for tea classification. Tea has been classified into (1) Assam type with the largest leaves, (2) China type with the smallest leaves and (3) Cambod with leaves of intermediate size.

The economic importance of the genus *Camellia* is attributed primarily to tea. Several books have been published describing the beneficial properties to health of tea (Kuroda and Hara 2004). Tea leaves contain more than 700 chemicals, among which the compounds closely related to human health are flavanoides, amino acids, vitamins (C, E and K), caffeine and polysaccharides. Moreover, 'tea drinking' has recently proven to be associated with cell-mediated immune function of the human body. Tea plays an important role in improving beneficial intestinal microflora, as well as providing immunity against intestinal disorders and in protecting cell membranes from oxidative damage. Tea also prevents dental caries due to the presence of fluorine. The role of tea is well established in normalizing blood pressure, lipid depressing activity, prevention of coronary heart diseases and diabetes by reducing the blood-glucose activity (Chen 1999). Tea also possesses germicidal and germistatic activities against various gram positive and gram negative human pathogenic bacteria (Chen

---

<sup>1</sup> Department of Plantation Crops and Processing, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar, 736165, West Bengal, India, e-mail: mondalTk@yahoo.com

1999). Both green and black tea infusions contain a number of antioxidants, mainly catechins that have anti-carcinogenic, anti-mutagenic and anti-tumor properties.

## 2 The Genome

The genome size in terms of 4C DNA for tea is  $15.61 \pm 1.06$ . Generally, tea chromosomes are small in size and tend to clump together due to 'stickiness'. Tea is generally diploid ( $2n = 30$ ,  $x = 15$ ) and its karyotype ranges from 1.28 to  $3.44 \mu$ . The  $r$  value (ratio of long to short arm) for all 15 pairs of chromosomes varies from 1.00 to 1.91. This consistency in diploid chromosome number suggests a monophyletic origin. However, triploid ( $2n = 45$ ) tea such as cvs. TV-29, HS-10 A and UPASI-3, tetraploids ( $2n = 60$ ), pentaploids ( $2n = 75$ ) and aneuploids ( $2n \pm 1$  to 29), have also been identified in natural tea populations (Zhan et al. 1987).

## 3 Tea Breeding

Sexual hybridization between two individuals and 'selection' among existing plants are the two major means of tea improvement, which has resulted in the development of more than 600 cultivated varieties today with better yield, quality or traits such as resistance to drought and diseases. However, the breeding objective of tea varies with region depending upon the production constraints prevailing in that region (Table 1).

Hybridization of tea is achieved by either natural or hand pollination. In natural hybridization, based on known characters such as previous performance of yield and quality or disease resistance, two parents are planted side by side in an isolated place and allowed to bear fruit. Subsequently, the seeds ( $F_1$ ) are harvested, raised and planted again. If average performance of these plants is found to be superior to that of either parent, then seeds are released as hybrid seed or bi-clonal seed. However, some of the outstanding performers among these progenies are marked, multiplied vegetatively and verified for multi-location trials. The best performer is then selected and released as a clone. Hand pollination or controlled crossing, despite being an important approach, has had limited success in tea breeding. This may be due to (1) the low success rate, (2) availability (2–3 months) of tea flowers for a short period, (3) the long period required for seed maturation (12–18 months) and (4) variation in flowering time for different clones. Only a few clones have been developed recently in Kenya and Malawi through hand pollination.

Selection is the most popular, age-old practice in tea breeding. Since commercial tea gardens were initially established with seeds, great variability exists



**Table 1.** Major production constraints of tea

Production constraint	Remarks
<i>Abiotic stress</i>	
Drought	Reduces productivity. Occurs in all tea-growing regions of the world
Winter dormancy	No leaf production during winter months. Occurs in south India, Japan and China, etc.
Hail	Causes economic loss, as young leaves during rainy season are mostly affected. Generally occurs in hilly regions
Waterlogging	Reduces productivity during rainy season. Generally occurs in north-east India
<i>Biotic stress</i>	
Diseases such as blister blight and stem canker	Blister blight causes severe damage, as only young leaves are infected. Generally occurs in Japan, Sri Lanka and south India
Pests such as red spider mite, tea mosquito bug and leaf sucking pest	Rest of the pests and diseases Generally occur in all tea-growing regions of the world

amongst the plants. Many elite plants were identified amongst existing bushes and released as clones. The majority of tea clones have been developed through selection, but their pedigrees remain unknown.

## 4 Tissue Culture

As *C. sinensis* is a woody perennial, conventional tea breeding is time-consuming and labour-intensive. Various constraints of tea breeding have been discussed previously (Mondal et al. 2004). These constraints may be circumvented by tissue culture techniques, although many attempts to exploit tissue culture for tea propagation and improvement have been unsuccessful (Table 2).

### 4.1 Micropropagation

Tea micropropagation, which comprises three different phases, and other tissue culture techniques have been reviewed previously (Kato 1989; Vieitez et al. 1992; Dood 1994; Das 2001; Mondal 2002; Mondal et al. 2004, 2005b). In the 1980s, the development of methods for tea micropropagation focused mainly on standardizing parameters, such as identification of suitable explants, overcoming microbial contamination, and the optimization of growth regulators in culture media to improve shoot proliferation. It is now widely accepted that nodal segments (0.5–1 cm) cultured on MS (Murashige and Skoog 1962) based

Table 2. Summary of different cell culture techniques for tea

Technique	Objective	Status	Remarks	Reference
Somaclonal and gamatoclonal variation	To develop a mutant resistant to different stresses	Few plants with drought tolerance observed <i>in vitro</i>	No success with commercial exploitation	Chen and Liao (1983); Rajkumar et al. (2001)
Artificial seed	Storage of propagules	Nodal segments and somatic embryos encapsulated	Maximum 60 days of storage was possible without reduction in germination	Mondal et al. (2000)
Protoplast culture	Production of haploid and hybrid plants	Fusion of two protoplasts has been achieved	No regeneration	Nakamura (1983); Purakayastha and Das (1994)
Anther culture	Production of haploid plants	Development of microcalli	No regeneration	Okano and Fuchinone, (1970); Shimokado et al. (1986); Raina and Iyer (1992)
Suspension culture	Production of secondary metabolites	Several secondary metabolites produced	Commercially not exploited	culture Forrest (1969); Ogutuga and Northcote (1970); Furuya et al. (1990); Matsuura et al. (1991); Hao et al. (1994)
Cryopreservation	Long-term storage of different propagules preservation in liquid nitrogen	Technique standardized for shoot tip	Not exploited further for either academic or commercial purposes	Chaudhury et al. (1990, 1991); Kuranuki and Yoshida (1991)

medium with benzyladenine (BA, 1–6 mg l<sup>-1</sup>) are optimal for shoot multiplication (Mondal et al. 2002a), while the presence of BA with either a concentrated (500 mg l<sup>-1</sup>) pulse treatment of auxin, such as indole-3-butyric acid (IBA), or a low dose with  $\alpha$ -naphthaleneacetic acid (1–2 mg l<sup>-1</sup>) was effective for *in vitro* rooting. Efforts in the 1990s were devoted to hardening of micro-shoots to improve survival during acclimatization. These resulted in the development of several non-conventional approaches, such as a carbon dioxide-enriched hardening chamber (Sharma et al. 1999), biological hardening (Pandey et al. 2000) and micrografting (Prakash et al. 1999; Mondal et al. 2005b). Micrografting has been a useful technique for hardening micropropagated shoots of woody plants. In tea, this technique also increased the survival rate to more than 90% of micropropagated shoots. In addition, it reduced the hardening time by 1 year due to faster growth rate of the micrografted shoots. At present, tea tissue culture research has focused mainly on an evaluation of field performance of micropropagated plants. In our laboratory, mature field-grown micropropagated tea plants grown under field conditions were evaluated in terms of overall yield and quality (Mondal et al. 2005a). It was found that the root volume of tissue culture-derived plants was greater than that of conventional vegetatively propagated plants in the nursery. This may be attributed to the former being treated with IBA to promote root induction, which may be responsible for improved root development in the field. We concluded that micropropagation should be used only when there is a need to produce the maximum number of plants in the shortest period from a limited source. The ultimate success of a tea micropropagation protocol is determined by plant performance in the field.

## 4.2 Somatic Embryogenesis

Plant regeneration from cultured cells and tissues via somatic embryogenesis has been considered as the most efficient tissue culture system because of its single-cell origin and the presence of a root system. In tea, somatic embryogenesis was first reported in the late 1980s, during which research was focused primarily on standardizing parameters such as genotype, seed maturity, media composition, growth regulators and physical culture conditions. This led to the development of several culture protocols for plant regeneration, including large-scale production of somatic embryos using bioreactors (Akula and Akula 1999; Mondal et al. 2004).

We have developed a protocol for somatic embryo formation from cotyledons and the embryogenic culture was maintained for more than 4 years (Mondal et al. 2001a). Somatic embryos were multiplied via secondary embryogenesis on MS medium with half nitrates but 300 mg l<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>, 2 mg l<sup>-1</sup> BAP and 0.2 mg l<sup>-1</sup> IBA (Mondal et al. 2001a). These embryos were later matured after being transferred to MS medium containing 4% maltose and 3 mg l<sup>-1</sup> trans-cinnamic acid for 4 weeks and germinated when cultured on MS with 1.5 mg l<sup>-1</sup>

GA<sub>3</sub> (Mondal et al. 2002b). Using this protocol, 3000 somatic embryo-derived plants were transferred to the field at the Research and Development Department, Tata Tea Limited, India (Mondal et al. 2004). Currently, these plants are being tested for their field performance.

Interestingly, induction of *in vivo* embryogenesis of tea could be achieved without using the conventional tissue culture media. In an attempt to germinate tea seeds under sterile conditions inside steel boxes containing moist sand for the use of protoplast isolation, we observed that the induction of embryogenesis occurred on the cotyledon surface of mature tea seeds under *in vivo* conditions at 28 °C (Mondal et al. 2001c).

## 5 Genetic Transformation

Although transgenic technology has considerable potential for tea improvement, there were no reports on transgenic tea until 2000. The initial challenge to standardize the protocol succeeded only in developing stable transformed callus (Matsumoto and Fukai 1998, 1999; Mondal et al. 1999). Somatic embryos were used as targets for genetic transformation mediated by *Agrobacterium tumefaciens*, resulting in healthy transgenic tea plants for the first time. Its transgenic nature was confirmed by polymerase chain reaction (PCR) and southern blot analysis (Mondal et al. 2001b). Transgenic plants were grown in the glasshouse, but transgene stability in these plants remains to be elucidated as tea plants usually take years to flower and set seed. Luo and Liang (2000) also reported transient expression of the  $\beta$ -glucuronidase (*gus*) gene in tea calli, but transgenic plants were not produced.

Attempts have also been made to transform tea using *A. rhizogenes*, which usually results in hairy root production. Zehra et al. (1996) reported the formation of hairy roots in tea inoculated with *A. rhizogenes* strain A4. The transformed roots were verified by mannopine analysis. In another study, the basal portions of 4 to 6-month-old *in vitro*-grown tea shoots were inoculated with the bacteria, followed by co-cultivation in liquid MS-based medium supplemented with IBA (5 mg l<sup>-1</sup>) and rifampicin (100 mg l<sup>-1</sup>) (Konwar et al. 1998). After 32–45 days of culture, roots were initiated from the basal portion of 66% of the explants. The main aim of the study was to enhance hardening of micropropagated tea shoots by inducing hairy roots at the basal portion. However, the hairy root culture has not been exploited to produce the tea secondary metabolites commercially, although tea is rich in alkaloids.

To date, transgenic tea plants have not been produced via biolistic bombardment, although there was a preliminary study showing transient gene expression following gene delivery by this procedure (Akula and Akula 1999). In this study, somatic embryos were bombarded with gold particles (1.5–3  $\mu$ m diameter) coated with the plasmid p2k7. Factors such as the distance between the site of microprojectile delivery and target tissue, helium pressure and the

state of the target tissue were optimized to obtain transient expression of the *gus* gene after 30–40 h of bombardment. Transient expression of the *gus* gene up to 1085 blue spots/shoot was obtained in bombarded somatic embryos. However, there was no report on the regeneration of transgenic plants from such bombarded somatic embryos. Despite the fact that transgenic technology offers considerable potential for tea improvement, the main aim of current tea research remains to develop an efficient transformation protocol.

## 6 Molecular Genetics

The progress of tea breeding has been slow due to the lack of reliable selection criteria. Since the release of the first commercial tea cv. TV-1 in 1923, several morphobiochemical markers have been used (Table 3). However, selection for desired agronomic traits using these markers shows low efficacy, owing to the fact that most traits are polygenic in nature. Furthermore, trait manifestation can be influenced markedly by environmental factors, which result in continuous variation, with a high degree of plasticity. Several isozymes, such as peroxidase and esterase, have also been used to analyze qualitative and quantitative variation among different species and cvs. of *Camellia* (Chengyin et al. 1992; Singh and Ravindranath 1994; Mondal et al. 2004). However, isozyme analysis in tea is limited to a few enzymes with inadequate polymorphism (Wachira et al. 1995).

DNA-based markers can be used to overcome some inherent problems encountered in morphobiochemical markers. Different DNA markers that have been used to characterize tea germplasm are presented in Table 4. Improved understanding of the genetic diversity in tea germplasm resource at the molecular level may facilitate (1) preservation of the intellectual property rights of tea breeders, (2) identification of individual tea cultivar by making a molecular passport, (3) prevention of duplicate entry of different genotypes in the tea gene pool, (4) efficient selection of the varieties for hybridization programs and composite plant production, (5) taxonomic classification of tea genotypes based on molecular markers, and (6) varietal improvement of tea for agronomically important traits through marker-assisted selection.

### 6.1 Random Amplified Polymorphic DNA (RAPD)

Since the first report on RAPD markers in Kenyan tea germplasm (Wachira et al. 1995), they have been used to analyze phylogenetic relationships amongst tea genotypes in different regions, including India (Mondal 2000; Mondal et al. 2000), Japan (Tanaka and Yamaguchi 1996; Chen and Yamaguchi 2002, 2005), China (Chen et al. 1998a, b, 1999, 2002a, b), South Africa (Wright et al. 1996), Taiwan (Lai et al. 2001), South Korea (Kaundun et al. 2000; Kaundun and Park 2002) and Portugal (Jorge et al. 2003)(Table 4). In addition,

**Table 3.** Morphochemical markers of tea

Criterion	Reference
Leaf geometry	Banerjee (1987)
Bush vigor	Barua and Dutta (1971)
Quantity and shape of scleroids	Barua (1958)
Leaf pose, color, serration of the margin and angle	Eden (1976)
Chlorophyll content/photosynthesis rate	Ghosh-Hazra (2001)
Quantitative changes in chlorophyll a and chlorophyll b and carotenoids	Hazarika and Mahanta (1984)
Epicuticular wax	Kabir et al. (1991)
Volatile flavor compound	Borse et al. (2002)
Dry matter production and partitioning	Magambo and Cannell (1981)
Green leaf catechin and ratio of dihydroxylated to trihydroxylated catechin	Magoma et al. (2000)
Root length	Nagarajah and Ratnasurya (1981)
Caffeine and volatile flavor compounds	Owuor and Obanda (1998)
Chloroform test	Sanderson (1964)
Polyphenol oxidase, individual polyphenols, presence or absence of certain phenolic and amino acids and chlorophyll content	Robertson (1992)
Pruning litter weight	Satyanarayan and Sharma (1982)
Anthocyanin pigmentation in young leaves	Satyanarayan and Sharma (1986)
Leaf, floral biology and growth morphology	Sealy (1958)
Evenness of flush, plucking density and recovery time from pruning.	Singh (1999)
Tarpen index	Takeo (1981)
Leaf pubescence	Wight and Barua (1954)
Phloem index	Wight and Barua (1954)

RAPD has been used to detect genetic fidelity in the *in vitro*-derived tea cv. T-78 (Mondal and Chand 2001) and, together with inter simple sequence repeat (ISSR) and restriction fragment length polymorphism (RFLP) markers, to evaluate genetic integrity of diploid and triploid microp propagated tea cvs. (Devarumath et al. 2002). Results showed reduced polymorphism in the nuclear genome compared to the mitochondrial genome; polymorphism was not detected in the chloroplast genome. It was concluded that variation amongst microp propagated plants is genotype dependent, rather than due to the culture conditions.

Mondal et al. (2000) described a simple method of DNA isolation from polyphenol-rich plants of eight different genera and 20 commercially important tea cultivars without using liquid nitrogen or phenol purification. The DNA was of sufficient quality to be used as a template for PCR amplification, indicating that the DNA isolation technique may facilitate RAPD analysis. Singh and Singh (2001) also reported a DNA isolation technique for RAPD

Table 4. Summary of molecular markers used in tea

Markers	Objectives	Reference
RAPD	Genetic diversity, characterization of micropropagated tea plants, varietal identification and genetic linkage maps	Wachira et al. (1995, 1997, 2001); Tanaka and Yamaguchi (1996); Wright et al. (1996); Chen et al. (1997, 1998a, b, 1999, 2002a, b, 2005a); Hackett et al. (2000); Kaundun et al. (2000); Mondal (2000); Mondal et al. (2000); Lai et al. (2001); Chen and Yamaguchi (2002); Devarumath et al. (2002); Kaundun and Parks (2002); Jorge et al. (2003); Beris et al. (2005)
ISSR	Genetic diversity	Lai et al. (2001); Mondal (2002)
AFLP	Genetic diversity	Chen et al. (1997); Paul et al. (1997); Rajasekaran (1997); Mishra and Sen-Mandi (2001); Wachira et al. (2001); Balasaravanan et al. (2003); Lee et al. (2003)
RFLP	Genetic diversity, development of species-specific probes, organization of 5srRNA, authentication of commercial tea samples	Matsumoto et al. (1994, 2002); Singh and Singh (2001); Dhiman and Singh (2003); Kaundun and Matsumoto (2003a, b)
SSR	Development and characterization of microsatellites	Ueno et al. (1999, 2000)
CAPS	Species-specific probes	Kaundun and Matsumoto (2003a)
Sequence analysis	Evaluation study, phylogenetic relationship	Prince and Parks (1997, 2000, 2001)



analysis from commercial black and green tea. The method has potential for testing the originality of commercial tea and for the identification of cvs. used by tea manufacturers for a particular brand.

## 6.2 Cleaved Amplified Polymorphic Sequence (CAPS)

Based on the sequence information of previously characterized tea genes, such as phenylalanine ammonia-lyase (PAL), chalcone synthase and dihydroflavonol 4-reductase, Kaundun and Matsumoto (2003a) reported CAPS markers for the analysis of 52 tea samples of diverse origin. Their results showed large differences amongst various types of tea and confirmed that the inheritance of the markers is co-dominant in nature.

## 6.3 Inter Simple Sequence Repeat (ISSR)

Twenty-five diverse Indian tea cvs. were analyzed using ISSR markers. A dendrogram was constructed using the unweighted pair group method analysis (UPGMA) method and revealed three distinct clusters of Cambod, Assam and China type, which concur with the known taxonomical classification of tea (Mondal 2002). ISSR markers have also been used to analyze the genetic diversity of 27 Taiwanese tea cultivars. (Lai et al. 2001). These results suggest that the ISSR-PCR method can be used for genetic fingerprinting and taxonomic classification of tea genotypes.

## 6.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP markers were used to assess the genetic variation of PAL in tea (Matsumoto et al. 1994, 2002). In combination with *Hind*III and *Eco*RV, a 2.3-kb probe was able to differentiate the Assam hybrids from Japanese green tea cvs. Furthermore, a study of the inheritance of the PAL gene in tea showed that this gene was present in a single copy per haploid genome and was inherited as a single gene following the Mendelian fashion. The results also led to the conclusion that Japanese green tea cvs. originated from a narrow genetic background.

## 6.5 Amplified Fragment Length Polymorphism (AFLP)

Paul and co-workers (1997) were the first to employ AFLP markers to detect genetic diversity and differentiation of different Indian and Kenyan tea clones. Later, AFLP markers were also employed to study genetic variation amongst 49 different south Indian (Balasaravanan et al. 2003) and 27 Darjeeling tea cvs. (Mishra and Sen-Mandi 2001), and the phylogenetic relationship among tea

cvs. from South Korea (Lee et al. 2003), China (Chen et al. 1997) and Japan (Wachira et al. 2001).

Hackett et al. (2000) reported the first genetic linkage map for tea, constructed from a mapping population thought to be derived from two non-inbred parents, using AFLP and RAPD markers. This map possessed 15 linkage groups of three or more markers, agreeing with the haploid chromosome number of tea. However, further analysis of non-inbred mapping populations using the statistical tool is required.

## 6.6 Simple Sequence Repeats (SSRs)

Although there has been no report regarding the development of microsatellites in tea, a study on SSRs has been reported in *C. japonica* (Ueno et al. 1999), a close relative of tea. Of 12 PCR primer pairs used, four resulted in a single-locus polymorphic amplification product. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters were calculated. Later, Ueno et al. (2000) investigated the spatial genetic structure of *C. japonica* using these four microsatellite primers. The spatial distribution of individuals was assessed to obtain an insight into spatial relationships between individuals and alleles. Morisita's index of dispersion plotted 518 individuals of *C. japonica* in a single clump and Moran's *I* spatial autocorrelation coefficient revealed weak genetic structure, indicating a low level of allele clustering among the individuals. Using the sequence information of these markers, Kaundun and Matsumoto (2002) developed microsatellite markers to study the genetic diversity of tea cultivars. Their results showed that genetic diversity assessed with nuclear microsatellites was more than that with chloroplast microsatellites, offering the prospect for the use of microsatellites in fingerprinting, mapping and population studies. The SSR primers that were characterized hitherto were derived from *C. japonica*. Recently, Freeman et al. (2004) developed 15 SSR primers which revealed a great variability across a wide range of tea clones.

## 6.7 Genomics

Genomics and its global expression profile (proteomics) offer a major advantage for rapid identification of genes and pathways that are associated with agronomically and economically important traits in plants. Multigenic characters, such as abiotic stress, drought and frost, which are the major production constraints in tea cultivation, can be studied in depth using these molecular approaches. This can be achieved, at least in part, by the development of a large number of expressed sequence tags (ESTs), and, to date, more than 1257 tea ESTs derived from a cDNA library prepared from young leaves of tea have been characterized (Park et al. 2004; Chen et al. 2005b). These ESTs are available in the NCBI GenBank database.

**Table 5.** Abundant ESTs found in the *C. sinensis* tender shoots cDNA library (Chen et al. 2005b)

Gene function	No. of ESTs
Ribosomal proteins	141
Arabidopsis-expressed proteins	85
Histones	78
Ribulose biphosphate carboxylase/oxygenase (Rubisco)	54
Chlorophyll a/b-binding proteins (CAB)	52
Metallothionein-like proteins	48
Photosystem II proteins	35
Photosystem I proteins	27
Early light-inducible proteins	19
Ubiquitin and polyubiquitin	18
Lipid transfer protein (LTP) family	18
Light-harvesting chlorophyll a/b binding proteins (Lhcb)	11
Zinc finger proteins	10
Auxin-binding proteins	9
Actinorhizal nodulin AgNOD-GHRP	9
Leucoanthocyanidin reductase	8
Cyclophilins	5
Dehydration-induced proteins	5

Apart from ESTs, full length genes have also been isolated from tea. Takeuchi et al. (1994) reported the isolation of chalcone synthase cDNA from Japanese green tea cv. 'Yabukuta'. Since then a large number of tea genes, some of which are presented in Table 5, have been sequenced and published in the database.

## 7 Conclusions

Micropropagation and somatic embryogenesis of tea have been studied extensively since the work of Forrest (1969) on tea callus culture. In order to improve the efficacy of tea tissue culture, efforts are being made to reduce the hardening time by various methods, such as carbon enrichment, biological hardening and micrografting, and a specially designed hardening chamber has been constructed to increase the survival rate of *in vitro*-grown plantlets. However, the commercial application of these tissue culture techniques remains a major challenge. In addition, shoot or plant regeneration from anthers and protoplasts is difficult in tea and related species. More effort should be devoted to overcoming the recalcitrance of anthers and protoplasts.

Transgenic plants serve as an important tool for the study of gene regulation, function, for crop improvement. Although transgenic plants expressing genes for desired traits have been reported for a wide range of crop species, the progress of tea transformation has been relatively slow. To date, tea has been

transformed with reporter genes, but transgenic tea expressing genes for agro-nomical traits has not been reported. This is due, at least in part, to a low transformation frequency. Future studies should focus on improvement of tea transformation by optimization of factors affecting transgenic plant production.

In tea, several molecular markers have been used for analysis of genetic diversity, phylogenetic relationship, genotyping, etc. and a low density linkage map is also available. However, a high density map has yet to be constructed, which may be achieved by an international tea consortium, to facilitate marker-assisted selection in order to identify agronomically useful genes.

## References

- Akula A, Akula C (1999) Somatic embryogenesis in tea (*Camellia sinensis* (L.) O. Kuntze. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, Dordrecht, pp 239–259
- Balasaravanan T, Pius PK, Kumar RR, Muraleedharan N, Shasany K (2003) Genetic diversity among south Indian tea germplasm (*Camellia sinensis*, *C. assamica* and *C. assamica* spp. *lasiocalyx*) using AFLP markers. *Plant Sci* 165:365–372
- Banerjee B (1987) Can leaf aspect affect herbivory? A case study with tea. *Ecology* 68:839–843
- Barua DN (1958) Leaf sclereids in tea taxonomy of the *Thea Camellias*. I. Wilson and related camellias. *Phytomorphology* 8:257–264
- Barua DN, Dutta KN (1971) Distribution of shoots in plucking surface of tea bush in relation to spacing. *Two Bud* 18:8–11
- Beris FS, Sandalli C, Canakci S, Demirbag Z, Belduz AO (2005) Phylogenetic analysis of tea clones (*Camellia sinensis*) using RAPD markers. *Biologia* 60:457–461
- Borse BB, Jagan Mohan RL, Nagalakshmi S, Krishnamurthy N (2002) Fingerprint of black teas from India: identification of the regio-specific characteristics. *Food Chem* 79:419–424
- Chaudhury R, Lakhanpal S, Chandel KPS (1990) Germination and desiccation tolerance of tea (*Camellia sinensis* (L.) O. Kuntze) seeds and feasibility of cryopreservation. *Sri Lankan J Tea Sci* 59:89–94
- Chaudhury R, Radhamani J, Chandel KPS (1991) Preliminary observation in the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* (L.) O. Kuntze) seeds for genetic conservation. *Cryo Lett* 12:31–36
- Chen L, Yamaguchi S (2002) Genetic diversity and phylogeny of tea plant (*Camellia sinensis*) and its related species and varieties in the section *Thea* genus *Camellia* determined by randomly amplified polymorphic DNA analysis. *J Horticult Sci Biotechnol* 77:729–732
- Chen L, Yamaguchi S (2005) RAPD markers for discriminating tea germplasms at the inter-specific level in China. *Plant Breed* 124:404–409
- Chen L, Chen D, Gao Q, Yang Y, Yu F (1997) Isolation and appraisal of genomic DNA from tea plant (*Camellia sinensis* (L.) O. Kuntze). *J Tea Sci* 17:177–181
- Chen L, Gao Q, Yang Y, Yu F, Chen D (1998a) Optimum amplification procedure and reaction system for RAPD analysis of tea plants (*Camellia sinensis* (L.) O. Kuntze). *J Tea Sci* 18:16–20
- Chen L, Yang Y, Yu F, Gao Q, Chen D (1998b) Genetic diversity of 15 tea (*Camellia sinensis* (L.) O. Kuntze) cultivars using RAPD markers. *J Tea Sci* 18:21–27
- Chen L, Yu F, Yang Y, Gao Q, Chen D, Xu C (1999) A study on genetic stability of excellent germplasm (*Camellia sinensis* (L.) O. Kuntze) using RAPD markers. *J Tea Sci* 19:13–16
- Chen L, Wang PS, Yamaguchi S (2002a) Discrimination of wild tea germplasm resources (*Camellia* sp.) using RAPD markers. *Agric Sci China* 1:1105–1110
- Chen L, Yamaguchi S, Wang PS, Xu M, Song WX, Tong QQ (2002b) Genetic polymorphism and molecular phylogeny analysis of section *Thea* based on RAPD markers. *J Tea Sci* 22:19–24

- Chen L, Gao QK, Chen D, Chang JX (2005a) The use of RAPD markers for detecting genetic diversity, relationship and molecular identification of Chinese elite tea genetic resources [*Camellia sinensis* (L.) O. Kuntze] preserved in a tea germplasm repository. *Biodiver Conserv* 14:1433–1444
- Chen L, Zhao LP, Gao QK (2005b) Generation and analysis of expressed sequence tags from the tender shoots cDNA library of tea plant (*Camellia sinensis*). *Plant Sci* 168:359–363
- Chen Z (1999) Pharmacological functions of tea. In: Jain NK (ed) *Global advances in tea sciences*. Aravali Books International, New Delhi, pp 333–358
- Chen Z, Liao H (1983) A success in bringing out tea plants from the anthers. *China Tea* 5:6–7
- Chengyin L, Weihua L, Mingjun R (1992) Relationship between the evolutionary relatives and the variation of esterase isozymes in tea plant. *J Tea Sci* 12:15–20
- Das SC (2001) Tea. In: Parthasarathy VA, Bose TK, Deka PC, Das P, Mitra SK, Mohandas S (eds) *Biotechnology of horticultural crops, vol. 1*. Naya Prokash, Calcutta, pp 526–546
- Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N, Raina S (2002) RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type). *Plant Cell Rep* 21:166–173
- Dhiman B, Singh M (2003) Molecular detection of cashew husk (*Anacardium occidentale*) adulteration in market samples of dry tea (*Camellia sinensis*). *Planta Med* 69:882–884
- Dood AW (1994) Tissue culture of tea (*Camellia sinensis* (L.) O. Kuntze) – a review. *Int J Trop Agric* 12:212–247
- Eden T (1976) *Tea*. Longman, London
- Forrest GI (1969) Studies on the polyphenol metabolism of tissue culture derived from the tea plant (*C. sinensis* L.). *Biochem J* 113:765–772
- Freeman S, West J, James C, Lea V, Mayes S (2004) Isolation and characterization of highly polymorphic microsatellites in tea (*Camellia sinensis*). *Mol Ecol Notes* 4:324–326
- Furuya T, Orihara T, Tsuda Y (1990) Caffeine and theanine from cultured cells of *Camellia sinensis*. *Phytochemistry* 29:2539–2547
- Ghosh-Hazra N (2001) Advances in selection and breeding of tea – a review. *J Plant Crop* 29:1–17
- Hackett CA, Wachira FN, Paul S, Powell W, Waugh R (2000) Construction of a genetic linkage map for *Camellia sinensis* (tea). *Heredity* 85:346–355
- Hao C, Wang Y, Yang S (1994) Effects of macroelements on the growth of tea callus and the accumulation of catechins. *J Tea Sci* 14:31–36
- Hazarika M, Mahanta PK (1984) Compositional changes in chlorophylls and carotenoids during the four flushes of tea in north-east India. *Ind J Food Agric* 35:298–303
- Jorge S, Pedroso MC, Neale DB, Brown G (2003) Genetic differentiation of Portuguese tea plant using RAPD markers. *HortScience* 38:1191–1197
- Kabir SE, Ghosh-Hazra N, Chaudhuri TC (1991) Performance of certain clones under the agro-climatic conditions of Darjeeling. *Tea Board India Tech Bull* 5:1–8
- Kato M (1989) *Camellia sinensis* L. (tea): in vitro regeneration. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry, vol. 7 Medicinal and aromatic plants II*. Springer, Berlin Heidelberg New York, pp 83–98
- Kaundun SS, Matsumoto S (2002) Heterologous nuclear and chloroplast microsatellite amplification and variation in tea, *Camellia sinensis*. *Genome* 45:1041–1048
- Kaundun SS, Matsumoto S (2003a) Development of CAPS markers based on three key genes of the phenylpropanoid pathway in tea, *Camellia sinensis* (L.) O. Kuntze and differentiation between *assamica* and *sinensis* varieties. *Theor Appl Genet* 106:375–383
- Kaundun SS, Matsumoto S (2003b) Identification of processed Japanese green tea based on polymorphism generated by STS-RFLP analysis. *J Agric Food Chem* 51:1765–1770
- Kaundun SS, Park Y-G (2002) Genetic structure of six Korean tea populations revealed by RAPD-PCR markers. *Crop Sci* 42:594–601
- Kaundun SS, Zhyvoloup A, Park Y-G (2000) Evaluation of genetic diversity among elite tea (*Camellia sinensis* var. *sinensis*) accessions using RAPD markers. *Euphytica* 115:7–16

- Konwar BK, Das SC, Bordoloi BJ, Dutta RK (1998) Hairy root development in tea through *Agrobacterium rhizogenes*-mediated genetic transformation. *Two Bud* 45:19–20
- Kuranuki Y, Yoshida S (1991) Cryopreservation of tea seeds and excised embryonic axes in liquid nitrogen. In: *Proc Int Symp on Tea Science*, Shizuoka, pp 419–420
- Kuroda Y, Hara Y (2004) Health effects of tea and its catechins. Kluwer, Dordrecht
- Lai J-A, Yang W-C, Hsiao J-Y (2001) An assessment of genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. *Bot Bull Acad Sin* 42:93–100
- Lee S, Kim J, Sano J, Ozaki Y, Okubo H (2003) Phylogenetic relationships among tea cultivars based on AFLP analysis. *J Fac Agric* 47:289–299
- Luo YY, Liang YR (2000) Studies on the construction of *bt* gene expression vector and its transformation in tea plant. *J Tea Sci* 20:141–147
- Magambo MJS, Cannell MGR (1981) Dry matter production and partition in relation to yield of tea. *Exp Agric* 17:33–38
- Magoma GN, Wachira FN, Obanda M, Imbuga M, Agong SG (2000) The use of catechins as biochemical markers in diversity studies of tea (*Camellia sinensis*). *Gene Resour Crop Eval* 47:107–114
- Matsumoto S, Fukai M (1998) *Agrobacterium tumefaciens* mediated gene transfer in tea plant (*Camellia sinensis*) cells. *Jpn Agric Res Quart* 32:287–291
- Matsumoto S, Fukai M (1999) Effect of acetosyringone application on *Agrobacterium*-mediated transfer in tea plant (*Camellia sinensis*). *Bull Natl Res Ins Veg Orn Shizuoka* 14:9–15
- Matsumoto S, Takeuchi A, Hayatsu M, Kondo S (1994) Molecular cloning of phenylalanine ammonia-lyase cDNA and classification of varieties and cultivars of tea plants (*Camellia sinensis*) using the tea PAL cDNA probes. *Theor Appl Genet* 89:671–675
- Matsumoto S, Kiriwa Y, Takeda Y (2002) Differentiation of Japanese green tea as revealed by RFLP analysis of phenyl-alanine ammonia-lyase DNA. *Theor Appl Genet* 104:998–1002
- Matsuura T, Kakuda T, Kinoshita T, Takeuchi N, Sasaki K (1991) Production of theanine by callus culture of tea. In: *Proc Int Symp on Tea Science*, Shizuoka, pp 432–436
- Mishra RK, Sen-Mandi S (2001) DNA finger printing and genetic relationship study of tea plants using amplified fragment length polymorphism (AFLP) technique. *Ind J Plant Genet Resour* 14:148–149
- Mondal TK (2000) Studies on RAPD marker for detection of genetic diversity, in vitro regeneration and *Agrobacterium*-mediated genetic transformation of tea (*Camellia sinensis*). PhD thesis, Utkal University, Bhubaneswar
- Mondal TK (2002) Detection of genetic diversity among the Indian tea (*Camellia sinensis*) germplasm by inter-simple sequence repeats (ISSR). *Euphytica* 128:307–315
- Mondal TK, Chand PK (2001) Detection of genetic instability among the micropropagated tea (*Camellia sinensis*) plants. In *Vitro Cell Dev Biol-Plant* 37:1–5
- Mondal TK, Bhattacharya A, Sood A, Ahuja PS (1999) An efficient protocol for somatic embryogenesis and its use in developing transgenic tea (*Camellia sinensis* (L.) O. Kuntze) for field transfer. In: Altman A, Ziv M, Izhar S (eds) *Plant biotechnology and in vitro biology in the 21st century*. Kluwer, Dordrecht, pp 101–104
- Mondal TK, Singh HP, Ahuja PS (2000) Isolation of genomic DNA from tea and other phenolic rich plants. *J Plant Crops* 28:30–34
- Mondal TK, Bhattacharya A, Ahuja PS (2001a) Induction of synchronous secondary embryogenesis of tea (*Camellia sinensis*). *J Plant Physiol* 158:945–951
- Mondal TK, Bhattacharya A, Ahuja PS, Chang PK (2001b) Transgenic tea [*Camellia sinensis* (L.) O. Kuntze cv. Kangra Jat] plants obtained by *Agrobacterium*-mediated transformation of somatic embryos. *Plant Cell Rep* 20:712–720
- Mondal TK, Bhattacharya A, Sharma M, Ahuja PS (2001c) Induction of in vivo somatic embryogenesis in tea (*Camellia sinensis*) cotyledons. *Curr Sci* 81:101–104
- Mondal TK, Bhattacharya A, Sood A, Ahuja PS (2002a) Propagation of tea (*Camellia sinensis* (L.) O. Kuntze) by shoot proliferation of alginate-encapsulated axillary buds stored at 4 °C. *Curr Sci* 83:941–944



- Mondal TK, Bhattacharya A, Sood A, Ahuja PS (2002b) Factors affecting germination and conversion frequency of somatic embryos of tea. *J Plant Physiol* 159:1317–1321
- Mondal TK, Bhattacharya A, Lashmikumaron M, Ahuja PS (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell Tissue Organ Cult* 76:195–254
- Mondal TK, Deka A, Deka PC (2005a) Tea. In: Parthasarathy VA, Chattopadhyay PK, Bose TK (eds) *Plantation crops*, vol I. Naya Udyog, Calcutta, pp 1–148
- Mondal TK, Parathiraj S, Mohan Kumar P (2005b) Micrografting-technique to shorten the hardening time of micropropagated shoots of tea [*Camellia sinensis* (L.) O. Kuntze]. *Sri Lanka J Tea Sci* 70:5–9
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–493
- Nagarajah S, Ratnasurya (1981) Clonal variability in root growth and drought resistance in tea (*Camellia sinensis*). *Plant Soil* 60:153–155
- Nakamura Y (1983) Isolation of protoplasts from tea plant. *Tea Res J* 58:36–37
- Ogutuga DBA, Northcote DH (1970) Caffeine formation in tea callus tissue. *J Exp Bot* 21:258–273
- Okano N, Fuchinone Y (1970) Production of haploid plants by anther culture of tea in vitro. *Jpn J Breed* 20:63–64
- Owuor PO, Obanda M (1998) The use of chemical parameters as criteria for selecting for quality in clonal black tea in Kenya: achievements, problems and prospects: a review. *Tea* 19:49–58
- Pandey A, Palni LMS, Bag N (2000) Biological hardening of tissue culture raised tea plants through rhizosphere bacteria. *Biotechnol Lett* 22:1087–1091
- Park JS, Kim JB, Hahn BS, Kim KH, Ha SH, Kim JB, Kim YH (2004) EST analysis of genes involved in secondary metabolism in *Camellia sinensis* (tea), using suppression subtractive hybridization. *Plant Sci* 166:953–961
- Paul S, Wachira FN, Powell W, Waugh R (1997) Diversity and genetic differentiation among population of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. *Theor Appl Genet* 94:255–263
- Prakash O, Sood A, Sharma M, Ahuja PS (1999) Grafting micropropagated tea (*Camellia sinensis* (L.) O. Kuntze) shoots on tea seedling – a new approach to tea propagation. *Plant Cell Rep* 18:137–142
- Prince LM, Parks CR (1997) Evolutionary relationships in the tea subfamily Theoiidae based on DNA sequence data. *Int Camellia J* 29:135–144
- Prince LM, Parks CR (2000) Estimation on relationships of Theoiidae (Theaceae) inferred from DNA data. *Int Camellia J* 32:79–84
- Prince LM, Parks CR (2001) Phylogenetic relationships of Theaceae inferred from chloroplast DNA sequence data. *Am J Bot* 88:2309–2320
- Purakayastha A, Das SC (1994) Isolation of tea protoplast and there culture. In: *Proc 32nd Tocklai Conf*, Tea Research Association, Tocklai Experimental Station, Jorhat, pp 34–35
- Raina SK, Iyer RD (1992) Multicell pollen proembryoid and callus formation in tea. *J Plant Crops* 9:100–104
- Rajasekaran P (1997) Development of molecular markers using AFLP in tea. In: Varghese JP (ed) *Molecular approaches to crop improvement*. In: *Proc Natl Sem on Molecular Approaches to Crop Improvement*, Kottayam, Kerala, pp 122–129
- Rajkumar R, Balasusaravanam S, Jayakumar D, Haridas V, Marimuthu S (2001) Physiological and biochemical feathers of field grown somaclonal variants of tea. *UPASI Tea Res Found Bull* 54:73–81
- Robertson A (1992) The chemistry and biochemistry of black tea production: the non-volatiles. In: Willson KC, Clifford MN (eds) *Tea: cultivation to consumption*. Chapman and Hall, London, pp 555–597
- Sanderson GW (1964) The chemical composition of fresh tea flush as affected by clone and climate. *Tea Q* 35:101–109
- Satyanarayan N, Sharma VS (1986) Tea (*Camellia* L. spp) germplasm in south India. In: Srivastava HC, Vatsya B, Menon KKG (eds) *Plantation crops: opportunity and constraints*. Oxford IBH Publishing, New Delhi, pp 173–179



- Satyanarayana N, Sharma VS (1982) Biometric basis for yield prediction in tea clonal selection. In: Proc PLACROSYM IV, 3–5 December 1981, Mysore, pp 237–243
- Sealy JR (1958) A revision of the genus *Camellia*. Royal Horticultural Society, London
- Sharma M, Sood A, Nagar PK, Prakash O, Ahuja PS (1999) Direct rooting and hardening of tea microshoots in the field. *Plant Cell Tissue Organ Cult* 58:111–118
- Shimokado T, Murata T, Miyaji Y (1986) Formation of embryoid by anther culture of tea. *Jpn J Breed* 36:282–283
- Singh D, Singh M (2001) Organization of 5s ribosomal RNA genes in tea (*Camellia sinensis*). *Genome* 44:143–146
- Singh HP, Ravindranath SD (1994) Occurrence and distribution of PPO activity in floral organs of some standard and local cultivars of tea. *J Sci Food Agric* 64:117–120
- Singh ID (1999) Plant improvement. In: Jain NK (ed) *Global advances in tea science*. Aravali Book International, New Delhi, pp 427–448
- Takeo T (1981) Variations in amounts of linalool and geraniol produced in tea shoots by mechanical injury. *Phytochemistry* 30:2149–2151
- Takeuchi A, Matsumoto S, Hayatsu M (1994) Amplification of  $\beta$ -tubulin cDNA from *Camellia sinensis* by PCR. *Bull Nat Res Inst Veg Orn Plant Tea* 7:13–20
- Tanaka JI, Yamaguchi S (1996) Use of RAPD markers for the identification of parentage of tea cultivars. *Bull Nat Res Inst Veg Orn Plant Tea* 9:31–36
- Ueno S, Yoshimaru H, Tomaru N, Yamamoto S (1999) Development and characterization of microsatellite markers in *Camellia japonica* L. *Mol Ecol* 8:335–336
- Ueno S, Tomaru N, Yoshimaru H, Manabe T, Yamamoto S (2000) Genetic structure of *Camellia japonica* L. in an old-growth evergreen forest, Tsushima, Japan. *Mol Ecol* 9:647–656
- Vieitez AM, Vieitez ML, Ballester A, Vieitez E (1992) Micropropagation of *Camellia* spp. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*, vol 19. High-tech and micropropagation III. Springer, Berlin Heidelberg New York, pp 361–387
- Wachira F, Tanaka J, Takeda Y (2001) Genetic variation and differentiation in tea (*Camellia sinensis*) germplasm revealed by RAPD and AFLP variation. *J Hortic Sci Biotechnol* 76:557–563
- Wachira FN, Waugh R, Hackett CA, Powell W (1995) Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. *Genome* 38:201–210
- Wachira FN, Powell W, Waugh R (1997) An assessment of genetic diversity among *Camellia sinensis* L. (cultivated tea) and its wild relatives based on randomly amplified polymorphic DNA and organelle specific STS. *Heredity* 78:603–611
- Wight W, Barua PK (1954) Morphological basis of quality in tea. *Nature* 173:630–631
- Wright LP, Apostolides Z, Louw AI (1996) DNA fingerprinting of clones. In: Whittle AM, Khumalo FRB (eds) *Proc 1st Regional Tea Res Sem*, Tea Research Institute, Blantyre, pp 44–50
- Zehra M, Banerjee S, Mathur AK, Kukreja AK (1996) Induction of hairy roots in tea (*Camellia sinensis* L.) using *Agrobacterium rhizogenes*. *Curr Sci* 70:84–86
- Zhan Z, Ke N, Chen B (1987) The cytology of tea clonal cultivars Fujian shuixian and their infertile mechanism. In: *Proc Int Tea Quality – Human Health Symp*, Tea Research Institute, Chinese Academy of Agricultural Science, Beijing

## III.4 Sugarcane

L.H. ZHANG<sup>1</sup>, L.X. WENG<sup>1</sup>, and Z.D. JIANG<sup>2</sup>

### 1 Introduction

Sugarcane is a widely cultivated monocotyledonous crop economically important in many countries and regions of the world. There are three cultivated species, namely *Saccharum officinarum*, *S. barberi* and *S. sinense*, and two wild species, *S. spontaneum* and *S. robustum*. The early cultivars were mostly clones of *S. officinarum*, which was a domesticated high-sugar-content species of *S. robustum* (Daniels and Roach 1987). Modern cultivars, which have increased yield and disease resistance compared to early cultivars, were generally derived by crossing *S. officinarum* with *S. spontaneum*, and then backcrossing the hybrids to *S. officinarum*, followed by intercrossing and selection (Berding and Roach 1987).

Sugarcane has been used primarily as a raw material for sugar production. Around 20 million ha of canes are grown annually worldwide (Haley and Suarez 2004). It accounts for nearly 80% of total world sugar production, which constituted 110,000 million tons in 2004/2005, while sugar beet provided the other 20% of the world's sugar (Haley and Suarez 2004). Major cane sugar exporters were Australia, Brazil, Colombia, Cuba, Guatemala, South Africa and Thailand. Collectively, these seven countries produced about 36% of the world's sugar (Haley and Bolling 2005). Among these two sugar-producing crops, sugarcane appears to be more cost effective, with unit costs ranging between 11.19 and 12.54 US cents per pound of sugar, which was only about half the cost of beet sugar production (Haley and Bolling 2005). Both sugar production and consumption have increased steadily by about 2.3% annually since 1994 (Haley and Bolling 2005).

Sugarcane may also have promising potential in biofuel production. Since 1985, Brazil has been using more than half of the canes harvested for ethanol production and produces about 13–15 billion liters of hydrous and anhydrous alcohols annually (Haley and Bolling 2005). As biofuel is an important technology for reducing CO<sub>2</sub> emissions, European production of ethanol for application as an automotive fuel has grown 4.5 times since 1993, namely from 47,500 tons in 1993 to 216,000 tons in 2001 (van Thuijl et al. 2003). It is noteworthy that more than 95% of the world's ethanol was produced from agricultural

---

<sup>1</sup> Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673, e-mail: lianhui@imcb.a-star.edu.sg

<sup>2</sup> Department of Plant Pathology, South China Agricultural University, Guangzhou 510642, China

Table 1. Transgenic sugarcanes with introduced traits

Phenotype	Transgene and promoter	Transformation method	Performance	Reference
Herbicide resistance	<i>bar</i> , Pubi-1	Microprojectile bombardment	Resistant to 6 g l <sup>-1</sup> glufosinate ammonium; field testing was not reported	Gallo-Meagher and Irvine (1996)
	<i>bar</i> , Pubi-1	<i>Agrobacterium tumefaciens</i>	Resistant to 2.5 g l <sup>-1</sup> glufosinate ammonium; field testing was not reported	Enríquez-Obregón et al. (1998)
	<i>bar</i> , Pubi-1	Microprojectile bombardment	Resistant to 60 mg m <sup>-2</sup> glufosinate ammonium; field testing was not reported	Falco et al. (2000)
	<i>bar</i> , Pubi-1	Microprojectile bombardment	Highly resistant to glufosinate ammonium (1.4 kg ha <sup>-1</sup> ) in field testing	Leibbrandt and Snyman (2003)
	<i>bar</i> , P35S	<i>Agrobacterium tumefaciens</i>	Resistant to 2.5 g l <sup>-1</sup> glufosinate ammonium; field testing was not reported	Manickavasagam et al. (2004)
Insect resistance	A truncated <i>cryIAb</i> gene, P35S	Electroporation	<i>CryIAb</i> concentration in transgenic line was in the range of 0.5–1.3 ng mg <sup>-1</sup> soluble proteins; resistant to sugarcane stem borer in field test	Arencibia et al. (1997, 1999)
	GNA, Pubi-1	Microprojectile bombardment	Resistant to Mexican rice borer, but showed no inhibitory effect on sugarcane stem borer based on insect feeding assay	Sétamou et al. (2002)
	The genes encoding Soybean Kunitz trypsin inhibitor (SKTI) and Bowman-Birk inhibitor (SBBi), Pubi-1	Microprojectile bombardment	Retardation of the growth of sugarcane stem borer in insect feeding trial; glasshouse test showed little resistance	Falco and Silva-Filho (2003)
	Synthetic <i>cryIAc</i> , Pubi-1	Microprojectile bombardment	<i>CryIAc</i> concentration in transgenic line was in the range 1.8–10.0 ng mg <sup>-1</sup> soluble proteins; resistant to sugarcane stem borer in insect feeding assay and in glasshouse test	Weng et al. (2006)

Table 1. (continued)

Phenotype	Transgene and promoter	Transformation method	Performance	Reference
Disease resistance	<i>albD</i> , Pubi-1	Microprojectile bombardment	AlbD concentration in resistant transgenic lines was in the range from 1–10 ng mg <sup>-1</sup> soluble proteins; no chronic infection symptoms in the glasshouse test	Zhang et al. (1999)
	The SrMV SCH CP gene, Pubi-1	Microprojectile bombardment	Most resistant plants contain reduced steady-state mRNA level of the transgene; several immune plants were identified in glasshouse trial	Ingelbrecht et al. (1999)
	The gene encoding the segment 9 ORF 1 of Fiji disease virus, Pubi-1	Microprojectile bombardment	The transgene transcript was not detected; one transgenic line showed enhanced resistance to Fiji disease in glasshouse trial	McQualter et al. (2004)

crops in 2003, mainly sugar-rich plants including sugarcane, sugar beet and corn (Berg 2004). Given the strong interest in fuel ethanol production worldwide, sugarcane, which is one of the most effective solar energy converters, may play an even more significant role as a raw material for production of environmentally friendly biofuels.

Modern molecular technologies have a major role to play in increasing the field performance and productivity of sugarcane. Encouraging progress has been made already in several areas that have either direct or indirect impact on molecular improvement and biotechnology of this crop. These areas include (1) developing molecular markers of important agricultural traits and mapping of quantitative trait loci (QTLs) (Dookun 1998; Aitken et al. 2005), (2) deciphering genome sequence information (Vettore et al. 2003; Asano et al. 2004; Vincentz et al. 2004), and (3) establishing methods and exploring various approaches in the genetic improvement of sugarcane. This chapter highlights and discusses the recent progress in sugarcane genetic engineering and the related technologies, as well as the existing challenges and future perspectives.

## 2 Sugarcane Genetic Transformation

Optimization of plant regeneration from cultured tissues and cells of sugarcane, construction of efficient vectors tailored for expression in sugarcane, availability of selectable marker and reporter genes, and defined adaptation of the existing transformation methods have contributed to establishing several useful protocols in the genetic transformation of sugarcane. The breakthrough in sugarcane transformation marked the first milestone in the journey of sugarcane molecular improvement. Currently, three approaches to sugarcane genetic transformation have been reported.

### 2.1 Electroporation

It was noticed three decades ago that application of short high-voltage electrical pulses could create reversible pores in cell membrane. The technique was subsequently optimized for delivery of DNA into the cells of microbes, animals and plants, respectively. Production of transformed sugarcane callus from electroporated protoplasts was demonstrated a decade ago (Rathus and Birch 1992). However, preparation of protoplasts and plant regeneration from electroporated protoplasts was found to be tedious and unproductive, with the result that protoplast electroporation was used infrequently for genetic improvement of sugarcane (Table 1). The bottleneck of regeneration was later overcome by a modified protocol of electroporation using intact sugarcane embryogenic cells, which proved to be successful in the regeneration of transformed plants (Arencibia et al. 1995).

## 2.2 Microprojectile Bombardment

The approach of accelerating DNA-coated particles into living cells was reported in the late 1980s as an alternative procedure to overcome the biological limitations of *Agrobacterium* and the difficulties associated with plant regeneration from protoplasts (Klein et al. 1987). The method was adopted for transformation of sugarcane. By coupling the Emu promoter to the neomycin phosphotransferase (*nptII*) gene, the first genetically transformed sugarcane plants were produced in 1992 (Bower and Birch 1992). Subsequently, the method was optimized to enhance cotransformation efficiency of reporter and selectable marker genes (Bower et al. 1996). As the method is simple to use and in general applicable to all the varieties capable of regeneration, it has since become the most widely employed procedure for the introduction of marker genes, as well as agronomically important genes for genetic improvement, into sugarcane (Table 1). The disadvantage of microprojectile bombardment, however, was the high copy number of the transgene and uncertainty of vector sequences integrated into the genome of sugarcane (Bower et al. 1996), which may inactivate certain sugarcane genes and complicate subsequent genetic characterization.

## 2.3 *Agrobacterium*-Mediated Gene Transfer

*Agrobacterium*-mediated genetic transformation has been used for the genetic improvement of many dicotyledonous plants since the 1980s. The advantages of *Agrobacterium* transformation include the defined insertion of a discrete segment of DNA into the recipient genome, with gene insertion occurring at only one or a few loci. In 1998, three laboratories in Cuba and Australia demonstrated simultaneously that it was possible to transform two sugarcane cultivars using *Agrobacterium* (Arencibia et al. 1998; Elliott et al. 1998; Enríquez-Obregón et al. 1998). Southern blotting analysis confirmed that each transgenic sugarcane line contained only one to two intact copies of the selectable marker gene (Enríquez-Obregón et al. 1998). Two types of explants were used for transformation, including sugarcane meristems (Enríquez-Obregón et al. 1998) and calli (Arencibia et al. 1998; Elliott et al. 1998).

## 3 Genetic Improvement

The key limiting factors affecting the productivity of current sugarcane varieties are the damage caused by weeds, insects and pathogens, with average annual losses estimated to be about 30% (Blackburn 1984). Until now, most sugarcane genetic engineering efforts have focused on enhancing resistance to biotic stress.

### 3.1 Herbicide Resistance

Over \$40 million is spent in the USA annually in controlling weeds in sugarcane plantations (Gallo-Meagher and Irvine 1996), highlighting that weed control is a costly and prominent problem for sugarcane industries. Although the potential spreading of herbicide resistance to other plants and grasses via pollination is an issue worthy of further cautious investigation, engineering of herbicide resistance in sugarcane could offer advantages, allowing destruction of weeds at any season with minimal effect on the crop. Several laboratories in the USA (Gallo-Meagher and Irvine 1996), Cuba (Enríquez-Obregón et al. 1998), Brazil (Falco et al. 2000), South Africa (Leibbrandt and Snyman 2003) and India (Manickavasagam et al. 2004), respectively, reported the production of transgenic sugarcane resistant to the commonly used herbicide Basta (also known as Buster), with glufosinate ammonium as the active component, by introducing the *bar* gene encoding phosphinotrycin acetyltransferase (Table 1). Most laboratories used the maize Ubi-1 promoter (PUBi-1) to direct the expression of the *bar* gene (Gallo-Meagher and Irvine 1996; Enríquez-Obregón et al. 1998; Falco et al. 2000; Leibbrandt and Snyman 2003), with the exception of one which used CaMV 35S promoter (Manickavasagam et al. 2004). Resistance appeared to be strong, as transgenic sugarcane plants showing strong resistance grew very well when Basta was applied at the recommended concentration to eliminate other weeds (Enríquez-Obregón et al. 1998; Leibbrandt and Snyman 2003; Manickavasagam et al. 2004). At least in one study, a field trial has been conducted with the herbicide-resistant transgenic sugarcanes, and stable herbicide resistance has been demonstrated over three rounds of vegetative propagation (Table 1; Leibbrandt and Snyman 2003).

### 3.2 Insect Resistance

A range of insect pests can damage sugarcane, accounting for more than 10% of world yield losses of the crop (Ricaud and Ryan 1989). The Lepidoptera insects, including sugarcane stem borer [*Diatraea saccharalis* (F.)] and Mexican rice borer [*Eoreuma loftini* (Dyar)], are the most important pests of this crop. Control of these insects is often very difficult and expensive because of the typical tunnel-feeding behavior of the insect larvae into sugarcane stems, which prevents effective contact of chemical insecticides with the target insects. Therefore, engineering of insect resistance by producing insect resistance protein or molecules in sugarcane stems appears to be a realistic approach to terminate potential damage caused by these pests.

Three types of insect resistance proteins have been explored in the engineering of insect resistance in sugarcane, namely the insecticidal crystal proteins (Cry) produced by *Bacillus thuringiensis*, proteinase inhibitors and lectin molecules of plant origins (Table 1). Based on their sequence diversities, Cry proteins can be grouped into more than 20 subclasses, which may have



different insecticidal spectra and potencies (Schnepf et al. 1998). Among them, the proteins belonging to the CryIA subclass show specific toxicity against *Lepidoptera* insects. These proteins, produced by *B. thuringiensis* as protoxins, are solubilized and activated in the midgut of insects by proteolysis. The activated toxins (60–70 kDa) bind to the membrane of midgut columnar cells and form ion channels, inducing osmotic lysis of the epithelium and, hence, resulting in the death of insects (Schnepf et al. 1998). Proteinase inhibitors are natural antimetabolic proteins produced by many plants, which can interfere with digestive processes in insects. Production of these metabolic inhibitors appears to constitute an effective defense strategy against insects in some plants (Larry and Shade 2002). Lectins are carbohydrate binding proteins that are abundant in seeds and storage tissues of some plants. It is not yet fully understood how lectins exert their toxicity against insects (Murdock and Shade 2002), but presumably they interfere with the essential biological functions of insects by specific binding to carbohydrates located on cell membranes, or by stimulating endocytosis of lectins that disrupt insect cells.

The first transgenic insect-resistant sugarcane was reported by a Cuban group in 1997, using electroporation (Arencibia et al. 1997). A truncated *cryIAb* gene encoding the active region of the *B. thuringiensis* insecticidal crystal protein was expressed in transgenic plants under the control of the CaMV 35S promoter. Although the expression of CryIAb was low (Table 1), several selected transgenic plants showed significant larvicidal activity (Arencibia et al. 1997). The subsequent field trial showed that elite transgenic lines reduced the incidence of internode infection by sugarcane stem borers (Arencibia et al. 1999). However, a low to middle level of internode infection was noticed even on the transgenic line expressing the highest amount of CryIAb (Arencibia et al. 1997, 1999), indicating the necessity to increase the expression level of the transgene. More recently, a synthetic *cryIAC* gene, in which the GC content was increased from the original 37% to 47% following the codon usage pattern of sugarcane, was introduced into sugarcane under the control of the maize Ubi-1 promoter (Pubi-1) using microprojectile bombardment. Quantitative analysis of 18 transgenic lines showed that they produced 1.8–10.0 ng mg<sup>-1</sup> total soluble protein (Weng et al. 2006), which is more than a 10-fold increase over *cryIAb* transgenic plants (Table 1). Insect feeding trials and glasshouse tests showed that the transgenic sugarcane lines highly expressing CryIAC were highly resistant to sugarcane stem borer infection, resulting in complete mortality of the inoculated insects within 1 week after inoculation (Weng et al. 2006).

The previous study showed that the toxicity of lectins against insects could be species specific (Murdock and Shade 2002). Similarly, the transgenic sugarcanes expressing a snowdrop lectin (*Galanthus nivalis* agglutinin, *GNA*) reduced the larval survival of the Mexican rice borer, but did not show noticeable deleterious effects on development and survival of insect larvae and pupae of sugarcane borer (Sétamou et al. 2002). The molecular mechanisms accounting for these differences remain to be elucidated.

The potential of two genes encoding soybean protease inhibitors, i.e., Kunitz trypsin inhibitor (SKTI) and Bowman-Birk inhibitor (SBBI), has been tested recently in transgenic sugarcane plants in conferring resistance against sugarcane borer in Brazil (Falco and Silva-Filho 2003). The genes were placed under the control of the Pubi-1 promoter and introduced into the genome of sugarcane by microprojectile bombardment. Insect feeding tests showed that larvae feeding on the transgenic sugarcane leaves containing SKTI and SBBI inhibitors were delayed in their development. However, these transgenic plants did not show significantly improved resistance when compared with the untransformed control in a glasshouse test (Falco and Silva-Filho 2003). The paper did not provide the quantitative data on protein expression of these inhibitors. Given the observation that 0.5% (w/w) of the semi-purified extract of soybean proteinase inhibitor in the artificial diet merely increased insect mortality by about one-fold (Pompermayer et al. 2001), it is estimated that the expression level of these protease inhibitors in transgenic sugarcane should be more than  $50 \text{ ng mg}^{-1}$  of total soluble proteins in order to confer a level of insect resistance of practical value.

### 3.3 Disease Resistance

Many viruses and bacterial and fungal pathogens can cause severe economic losses in sugarcane production. Three studies discussed below suggest promising potential in the engineering of disease resistance, based on a normal understanding of the mechanisms of infection and the principles of the host disease resistance.

Sugarcane leaf scald disease, induced by *Xanthomonas albicidins*, can cause severe economic losses (Ricaud and Ryan 1989). There are two forms of leaf scald, namely the chronic phase and the acute phase. The typical symptom of chronic *X. albicidins* infection is the presence on the leaf lamina of 'white-pencil-line' streaks about 1–2 mm wide following the direction of the main veins. The acute phase is characterized by a sudden wilting and death of mature stalks; the acute symptom normally occurs only following a period of stress, especially prolonged dry weather or dry weather following a wet period (Ricaud and Ryan 1989). Several lines of evidence showed that albicidin (Alb), a phytotoxin and antibiotic produced by *X. albicidins*, played a key role in causing the white-pencil-line symptom, because the Alb<sup>-</sup> mutants, generated either by transposon mutagenesis (Birch and Patil 1987) or by expression of an Alb detoxification enzyme encoded by *albD* in wild type *X. albicidins* (Zhang and Birch 1997), failed to induce any white-pencil-line disease symptoms. However, the mechanism of acute phase infection is not fully understood.

The impact of the AlbD enzyme, which was characterized as an albicidin hydrolase (Zhang and Birch 1997), on leaf scald resistance was evaluated by generating *albD* transgenic sugarcane plants. Glasshouse tests showed that the plants with albicidin detoxification capacity equivalent to 1–10 ng of AlbD en-

zyme per milligram of leaf protein did not develop white-pencil-line symptoms in the inoculated leaves, whereas all untransformed control plants developed severe symptoms (Zhang et al. 1999). Moreover, there was a strong negative correlation between AlbD activity and pathogen multiplication in the inoculated leaves, suggesting that albicidin resistance due to *albD* expression in transgenic sugarcane can substantially reduce *X. albicidins* multiplication in the primary infection (Zhang et al. 1999).

The sugarcane mosaic virus (SCMV) complex comprises four to five different potyviruses, which have a monopartite, positive-strand RNA genome. Members of the SCMV complex can cause mosaic symptoms and yield loss in susceptible members of sugarcane, maize, sorghum and other poaceous plants. The coat protein (CP) gene of sorghum mosaic potyvirus (SrMV), a member of the SCMV, was used as a transgene under the control of the Pubi-1 promoter to engineer sugarcane SCMV resistance (Ingelbrecht et al. 1999). Most resistant plants showed low or undetectable steady-state CP transgene mRNA levels. Increased DNA methylation was observed in the transcribed region of the CP transgene, suggesting that the CP transgene enhanced virus resistance by activating the posttranscriptional gene silencing mechanism in transgenic sugarcane (Ingelbrecht et al. 1999). Interestingly, transgene-conferred virus resistance requires a high degree of sequence similarity between the transgene and the invading virus. When the SrMV CP transgenic sugarcane plants were challenged with the SrMV virus, the majority of the transgenic plants did not show any symptoms. However, when challenged with SCMV, which shares about 75% sequence homology with SrMV in the CP nucleotide sequences, most of these SrMV-resistant plants showed similar symptoms to the untransformed control plants (Ingelbrecht et al. 1999).

Fiji disease of sugarcane, caused by Fiji disease virus (FDV), is the most important viral disease affecting the Australian sugar industry. Typical symptoms of Fiji disease include raised whitish galls on the abaxial side of the leaf lamina, and severe infection can cause stunting and death of the apical meristem. FDV is a dsRNA virus consisting of 10 linear segments ranging in size from about 1.8–4.4 Kb (Mertens et al. 2000). The gene encoding a structural protein of unknown function located at segment 9 ORF1 was cloned under the control of Pubi-1 promoter and transformed into sugarcane by microprojectile bombardment (McQualter et al. 2004). Of the 64 transgenic lines tested, one showed significantly enhanced resistance to Fiji disease in glasshouse trials. However, it is not clear at this stage what the mechanism of resistance is and, hence, the contribution of the transgene to the observed virus resistance, because transcripts of the transgene were not detected in either susceptible or resistant transgenic sugarcanes by Northern blotting analysis (McQualter et al. 2004). The production of more transgenic sugarcane lines would be beneficial to confirm the potential of this transgene in conferring resistance to FDV disease in sugarcane.

## 4 Factors Affecting Transgene Expression and Performance

Transgene expression level could influence directly the field performance of transgenic sugarcane plants because, in most cases, the product of the transgene should be expressed at an adequate concentration to achieve the expected biological activity. Several factors may influence the expression of transgenes, including promoter strength, the codon usage of transgenes and gene silencing.

### 4.1 Promoter Strength

Among the several promoters tested, the rice polyubiquitin promoter RUBQ2 appears to be the strongest promoter in sugarcane, followed by Pubi-1 promoter, which has been widely used in transgenic sugarcane (Table 1). Quantification analysis of transgenic sugarcane plants derived from microprojectile bombardment showed that stable GUS activity by RUBQ2 was 1.6-fold higher than that by the maize Ubi-1 promoter, whereas GUS activity was not detected in transgenic plants with the CaMV 35S promoter (Liu et al. 2003). Since transient expression of GUS under the CaMV 35S promoter was also hardly detectable (Liu et al. 2003), the failure of *gus* gene expression under the same promoter in stable transgenic sugarcane was unlikely to be due to multi-copy transgene-induced silencing.

### 4.2 GC Content and Codon Usage

The GC content of coding regions, and hence the codon usage patterns of different organisms, can be highly variable. Genome sequence analysis revealed that there are remarkable divergences in nucleotide composition among different organisms (Tredj et al. 2002; Ou et al. 2003). Significantly, many microorganisms are low in GC content, while multicellular eukaryotes are, in general, GC-rich. In particular, the GC content of sugarcane genes is up to 55%, which is about 19% higher than those of the soil bacterium *Bacillus thuringiensis* ([www.kazusa.or.jp/codon/](http://www.kazusa.or.jp/codon/)). Evidence suggests that GC-rich regions are more active in transcription in plants and animals because their superior bendability and B-Z transition ability favor open chromatin conformation, whereas AT-rich regions attract chromatin condensation that hinders transcription (Herbert and Rich 1999; Vinogradov 2003). Consistently, it was shown recently that by increasing the GC content of the *cryIAc* gene of *B. thuringiensis* from 37% to 47% increased its stable expression in transgenic sugarcane by at least two- to three-fold (Weng et al. 2006).

### 4.3 Gene Silencing

A realistic concern for commercialization of transgenic sugarcane is whether transgenes will be stably expressed in consecutive generations in the field, in

particular, for transgenic plants carrying multiple copies of transgenes. It has been well documented in dicotyledons that homology-dependent gene silencing can occur between homologous transgenes (Vaucheret et al. 1998). Gene silencing can occur through any one of two distinct epigenetic processes, one operating at the transcriptional level and one at the posttranscriptional level (Vaucheret et al. 1998). The posttranscriptional gene silencing phenomenon was documented in transgenic sugarcane carrying the sorghum mosaic potyvirus strain SCH coat protein (CP) gene (Ingelbrecht et al. 1999). Increased DNA methylation was observed in the CP transcripts of transgenic plants. Nevertheless, such a posttranscriptional gene silencing modification of CP transcripts was positively correlated with virus resistance, because the CP transcripts alone, but not the CP proteins, could confer virus resistance by triggering gene silencing mechanisms against the virus (Ingelbrecht et al. 1999). Another study on sugarcane transformed with the *bar* gene, which encodes resistance to the herbicide Buster (glufosinate ammonium), did not reveal any noticeable gene silencing effect (Leibbrandt and Snyman 2003). Field testing showed that the *pat* gene was stably expressed during three rounds of vegetative propagation. It is interesting to note that the GC content of the CP gene (NCBI Accession no. NC004035) is about 45%, while that of the *bar* gene (NCBI Accession no. AF404854) is up to 68%, which is closer to the GC content of sugarcane genes. More investigations should be carried out on the expression patterns of transgenes with different GC contents in transgenic sugarcane plants following propagation.

#### 4.4 Somaclonal Variation

A most common approach in plant genetic engineering is to introduce the desirable transgene(s) into elite commercial varieties or elite breeding lines. Commercial exploitation of transgenic plants therefore depends not only on adequate expression of the transgene(s), but also on maintenance of all other excellent traits of the elite varieties. This is particularly meaningful for sugarcane, in which the chance of backcrosses to restore the original genotype is limited by the complex ploidy of the plant. Evaluation of five selected transgenic lines expressing *cryIAb* showed that the majority of agronomic and industrial traits were maintained, but a small number of qualitative traits, such as disease resistance, were changed in the progeny clones (Arencibia et al. 1999). Genome polymorphism analyses using amplified fragment length polymorphism (AFLP) and random amplified microsatellite polymorphism (RAMP) identified three distinct groups of polymorphic band. Fifteen bands appeared in both transgenic progenies and untransformed control cell lines, three bands appeared only in untransformed control cell lines, and 33 bands appeared only in transgenic progenies (Arencibia et al. 1999). The data suggest the existence of not only cell culture-induced somaclonal variation, but also transformation-associated variation. AFLP analysis of three transgenic sugarcane populations

produced by *Agrobacterium tumefaciens* infection also enables a similar conclusion to be reached, that transformation was accompanied by limited, but detectable, genomic changes (Carmona et al. 2005).

## 5 Conclusions and Perspectives

Rapid advances in genetic transformation technologies since the early 1990s have opened up new possibilities for the molecular improvement of sugarcane, a monocotyledonous plant that produces the most biomass per hectare than any other annual crop. In comparison with direct gene delivery methods, such as microprojectile bombardment, *Agrobacterium*-mediated transformation has been reported in only a limited member of sugarcane varieties. It could be worthwhile to continue investigations on this technology and to broaden the spectrum of transformation as it offers excellent advantages, including low copy insertion of transgenes and the possibility of distinct integration of large-sized DNA into the sugarcane genome. Moreover, a better understanding of the factors affecting transgene expression, as well as the impacts of tissue culture, transformation protocol and transgene on somaclonal variation and global gene expression profiles of transgenic plants, would lead to more efficient molecular breeding process and the commercial exploitation of biotechnology.

The progress in the last decade also highlights the importance of basic research. In better understanding the infection mechanisms of insects and pathogens and sugarcane defense mechanisms, it will be possible to identify more useful transgenes and to engineer enduring and sustainable plant resistance to different biostresses. The efforts in determining the transcribed sugarcane genome sequence and, hopefully, in the near future the whole genome sequence, will be extremely important to our understanding of the molecular mechanisms of sucrose production and accumulation, biomass growth, solar energy conversion and biostress resistance.

The first phase of sugarcane genetic improvement using transgenic approaches concentrated on genetic engineering for biostress resistance. This is partly due to the availability of suitable transgenes and, partly, because that biostress resistance could be improved by introducing a single gene, which is technically more realistic. Nevertheless, knowledge and experiences obtained from these research and development activities would definitely benefit the subsequent genetic improvement of sugarcane in other respects, such as increasing sucrose yield and using transgenic sugarcane to produce value-added novel products. As sugarcane can produce and accumulate large quantities of carbohydrates, it would be convenient to engineer sugarcane to produce novel carbohydrates, in particular, sucrose metabolites. One good example may be isomaltulose and its derivatives (Zhang et al. 2002, 2003; Wu and Birch 2005), which have potential as functional sugars for human consumption. A similar



exciting perspective is to use genetic engineering to optimize the potential of sugarcane for biofuel production. Moreover, with the progress in transformation technologies, several genes, or a DNA region encoding a whole metabolic pathway, could be introduced into sugarcane for engineering of multiple biostress resistance traits, or for production of useful compounds and products.

## References

- Aitken KS, Jackson PA, McIntyre CL (2005) A combination of AFLP and SSR markers provides extensive map coverage and identification of homo(eo)logous linkage groups in a sugarcane cultivar. *Theor Appl Genet* 110:789–801
- Arencibia A, Molina PR, Delariva G, Selmanhousein G (1995) Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. *Plant Cell Rep* 14:305–309
- Arencibia A, Vázquez RI, Prieto D, Téllez P, Carmona ER, Coego A, Hernández L, De la Riva GA, Selman-Housein G (1997) Transgenic sugarcane plants resistant to stem borer attack. *Mol Breed* 3:247–255
- Arencibia AD, Carmona ER, Téllez P, Chan MT, Yu SM, Trujillo LE, Oramas P (1998) An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Res* 7:213–222
- Arencibia AD, Carmona ER, Cornide MT, Castiglione S, O'Reilly J, Chinae A, Oramas P, Sala F (1999) Somaclonal variation in insect-resistant transgenic sugarcane (*Saccharum* hybrid) plants produced by cell electroporation. *Transgenic Res* 8:349–360
- Asano T, Tsudzuki T, Takahashi S, Shimada H, Kadowaki K (2004) Complete nucleotide sequence of the sugarcane (*Saccharum officinarum*) chloroplast genome: a comparative analysis of four monocot chloroplast genomes. *DNA Res* 11:93–99
- Berding N, Roach BT (1987) Germplasm collection, maintenance, and use. In: Heinz DJ (ed) *Sugarcane improvement through breeding*. Elsevier Press, Amsterdam, pp 143–210
- Berg C (2004) World fuel ethanol analysis and outlook. <http://www.distill.com/world-fuel-ethanol-a&o-2004.html>
- Birch RG, Patil SS (1987) Evidence that an albicidin-like phytotoxin induces chlorosis in sugarcane leaf scald disease by blocking plastid DNA replication. *Physiol Mol Plant Pathol* 30:207–214
- Blackburn F (1984) *Sugarcane*. Longman, New York
- Bower R, Birch RG (1992) Transgenic sugarcane plants via microprojectile bombardment. *Plant J* 2:409–416
- Bower R, Elliott AR, Bernard AMP, Birch RG (1996) High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers. *Mol Breed* 2:239–249
- Carmona ER, Arencibia AD, Lopez J, Simpson J, Vargas D, Sala F (2005) Analysis of genomic variability in transgenic sugarcane plants produced by *Agrobacterium tumefaciens* infection. *Plant Breed* 124:33–38
- Daniels J, Roach BT (1987) Taxonomy and evolution. In: Heinz DJ (ed) *Sugarcane improvement through breeding*. Elsevier, Amsterdam, pp 7–84
- Dookun A (1998) Biotechnology for sugarcane. *Ag Biotech Net* 10:75N–80N
- Elliott AR, Campbell JA, Brettell RIS, Grof CPL (1998) *Agrobacterium*-mediated transformation of sugarcane using GFP as a screenable marker. *Aust J Plant Physiol* 25:739–743
- Enriquez-Obregón GA, Roberto I, Vázquez-Padrón DL, Prieto-Samsonov GA, De la Riva, Selman-Housein G (1998) Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. *Planta* 206:20–27



- Falco MC, Silva-Filho MC (2003) Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. *Plant Physiol Biochem* 41:761–766
- Falco MC, Neto AT, Ulian EC (2000) Transformation and expression of a gene for herbicide resistance in a Brazilian sugarcane. *Plant Cell Rep* 19:1188–1194
- Gallo-Meagher M, Irvine JE (1996) Herbicide resistant transgenic sugarcane plants containing the *bar* gene. *Crop Sci* 36:1367–1374
- Haley S, Bolling C (2005) USDA projects steady beet sugar production but lower cane sugar production. Electronic Outlook Report from the Economic Research Service ([www.ers.usda.gov](http://www.ers.usda.gov)). Sugar and Sweeteners Outlook sss-242, Economic Research Service, USDA, Washington, DC
- Haley S, Suarez NR (2004) USDA establishes fiscal year 2005 sugar marketing allotments. Electronic Outlook Report from the Economic Research Service ([www.ers.usda.gov](http://www.ers.usda.gov)). Sugar and Sweeteners Outlook sss-241, Economic Research Service, USDA, Washington, DC
- Herbert A, Rich A (1999) Left-handed Z-DNA: structure and function. *Genetica* 106:37–47
- Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploidy genome. *Plant Physiol* 119:1187–1197
- Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327:70–73
- Larry LM, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. *J Agric Food Chem* 50:6605–6611
- Leibbrandt NB, Snyman SJ (2003) Stability of gene expression and agronomic performance of a transgenic herbicide-resistant sugarcane line in South Africa. *Crop Sci* 43:671–677
- Liu DW, Oard SV, Oard JH (2003) High transgene expression levels in sugarcane (*Saccharum officinarum* L.) driven by the rice ubiquitin promoter RUBQ2. *Plant Sci* 165:743–750
- Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S (2004) *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. *Plant Cell Rep* 23:134–143
- McQualter RB, Dale JL, Harding RM, McMahon JA, Smith GR (2004) Production and evaluation of transgenic sugarcane containing a *Fiji disease virus* (FDV) genome segment S9-derived synthetic resistance gene. *Aust J Agric Res* 55:139–145
- Mertens P, Arella M, Attoui H, Belloncik S, Bergoin M, Boccardo G, Booth T, Chiu W, Diprose J, Duncan R (2000) Reoviridae. Virus taxonomy: the classification and nomenclature of viruses. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeogh DJ, Pringle CR, Wickner RB (eds) *The 7th Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego, pp 395–480
- Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. *J Agric Food Chem* 50:6605–6611
- Ou HY, Guo FB, Zhang CT (2003) Analysis of nucleotide distribution in the genome of *Streptomyces coelicolor* A3 (2) using the Z curve method. *FEBS Lett* 540:188–194
- Pompermayer PAR, Lopes WR, Terra JRP, Parra MCF, Silva-Filho MC (2001) Effects of soybean proteinase inhibitor on development, survival and reproductive potential of the sugarcane borer, *Diatraea saccharalis*. *Entomol Exp Appl* 99:79–85
- Rathus C, Birch RG (1992) Optimization of conditions for electroporation and transient expression of foreign genes in sugarcane protoplasts. *Plant Sci* 81:65–74
- Ricaud C, Ryan CC (1989) Leaf scald. In: Ricaud C, Ryan CC, Egan BT, Gillaspie Jr AG, Hughes CG (eds) *Diseases of sugarcane*. Elsevier, Amsterdam, pp 39–53
- Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62:775–806
- Sétamou M, Bernal JS, Legaspi JC, Mirkov TE, Legaspi BC (2002) Evaluation of lectin-expressing transgenic sugarcane against stalkborers (Lepidoptera: Pyralidae): effects on life history parameters. *J Econ Entomol* 95:469–477

- Tredj T, Yeramian E, Dujon B (2002) Amino acid composition of genomes, life styles of organism and evolutionary trends: a global picture with correspondence analysis. *Gene* 297:51–60
- van Thuijl E, Roos CJ, Beurskens LWM (2003) An overview of biofuel technologies, markets and policies in Europe. ECN-C-03-008. Energy Research Centre of the Netherlands, Amsterdam, p 5
- Vaucheret H, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. *Plant J* 16:651–659
- Vettore AL, da Silva FR, Kemper EL, Souza GM, da Silva AM, Ferro MIT, Henrique-Silva F, Giglioti EA, Lemos MVE, Coutinho LL, Nobrega MP, Carrer H, Franca SC, Bacci M, Goldman MHS, Gomes SL, Nunes LR, Camargo LEA, Siqueira WJ, Van Sluys MA, Thiemann OH, Kuramae EE, Santelli RV, Marino CL, Targon MLPN, Ferro JA, Silveira HCS, Marini DC, Lemos EGM, Monteiro-Vitorello CB, Tambor JHM, Carraro DM, Roberto PG, Martins VG, Goldman GH, de Oliveira RC, Truffi D, Colombo CA, Rossi M, de Araujo PG, Sculaccio SA, Angella A, Lima MMA, de Rosa VE, Siviero F, Coscrato VE, Machado MA, Grivet L, Di Mauro SMZ, Nobrega FG, Menck CFM, Braga MDV, Telles GP, Cara FAA, Pedrosa G, Meidanis J, Arruda P (2003) Analysis and functional annotation of an expressed sequence tag collection for tropical crop sugarcane. *Genome Res* 13:2725–2735
- Vincenz M, Cara FAA, Okura VK, da Silva FR, Pedrosa GL, Hemerly AS, Capella AN, Marins M, Ferreira PC, Franca SC, Grivet L, Vettore AL, Kemper EL, Burnquist WL, Targon MLP, Siqueira WJ, Kuramae EE, Marino CL, Camargo LEA, Carrer H, Coutinho LL, Furlan LR, Lemos MVE, Nunes LR, Gomes SL, Santelli RV, Goldman MH, Bacci M, Giglioti EA, Thiemann OH, Silva FH, Van Sluys MA, Nobrega FG, Arruda P, Menck CFM (2004) Evaluation of monocot and eudicot divergence using the sugarcane transcriptome. *Plant Physiol* 134:951–959
- Vinogradov AE (2003) DNA helix: the importance of being GC-rich. *Nucleic Acids Res* 31:1838–1844
- Weng LX, Deng HH, Xu JL, Li Q, Wang LH, Jiang ZD, Zhang HB, Li QW, Zhang LH (2006) Regeneration of sugarcane elite breeding lines and engineering of strong stem borer resistance. *Pest Manag Sci* 62:178–187
- Wu L, Birch RG (2005) Characterization of the highly efficient sucrose isomerase from *Pantoea dispersa* UQ68J and cloning of the sucrose isomerase gene. *Appl Environ Microbiol* 71:1581–1590
- Zhang D, Li X, Zhang LH (2002) Isomaltulose synthase from *Klebsiella* sp. strain LX3: gene cloning and characterization and engineering of thermostability. *Appl Environ Microbiol* 68:2676–2682
- Zhang D, Li N, Swaminathan K, Zhang LH (2003) A motif rich in charged residues determines product specificity in isomaltulose synthase. *FEBS Lett* 534:151–155
- Zhang LH, Birch RG (1997) The gene for albicidin detoxification from *Pantoea dispersa* encodes an esterase and attenuates pathogenicity of *Xanthomonas albilineans* to sugarcane. *Proc Natl Acad Sci USA* 94:9984–9989
- Zhang LH, Xu JL, Birch RG (1999) Engineered detoxification confers resistance against a pathogenic bacterium. *Nat Biotechnol* 17:1021–1024

# Subject Index

- Absciscic acid (ABA) 58  
*Acacia* 407  
– *mangium* 421, 425  
*Acaulospora melleae* 486  
acclimatization 175  
ACC  
  (1-aminocyclopropane-1-carboxylate)  
  synthase 6, 7, 25, 45, 251, 259, 335  
ACC oxidase 6, 7, 25, 60, 87, 100, 101, 119,  
  220, 224, 226, 228, 251, 252, 259, 335, 339  
acetosyringone 22, 180, 291, 314, 435  
achene 316  
acid phosphatase 7  
*Actinidia* 329  
 $\text{AgNO}_3$  (See silver nitrate)  
*Agrobacterium* 242, 248–250, 287, 291,  
  292, 300, 362, 421, 434, 435, 487, 488, 508  
– *A. rhizogenes* 434, 442, 448  
– *A. tumefaciens* 38, 180, 391, 421, 434,  
  435, 440, 442, 448, 487, 548  
– T-DNA 291  
*Agrobacterium*-mediated transformation  
  21, 22, 77, 79, 84, 85, 89, 110, 113, 142,  
  223, 248, 287, 291, 300, 313, 372, 373, 421,  
  487, 488  
agronomic improvement 111  
alcohol acyltransferase 10  
allergens 253, 255, 261, 262, 267, 332  
*Allocasuarina* 441  
allotetraploid 222  
allotriploids 222  
alternate oxidase 65  
American black walnut 351  
 $\beta$ -amylase 8  
*Ananas* 98  
– *A. ananassoides* 98  
– *A. comosces* 98, 99, 112  
– var. *comosces* 98, 108, 112  
– var. *ananassoides* 98, 108  
– var. *bracteatus* 98, 108  
– var. *erectifolius* 99  
– var. *paraquazensis* 98  
– *A. nacruidibes* 98  
androgenesis 20, 485  
androsterility 150, 155  
– pEND1::BARNASE 150  
anthers 15, 21, 80, 84, 151, 190, 312, 356,  
  476, 484, 485, 530  
antibodies 25, 196, 246, 375, 376, 377  
*APETALA1* 43, 62, 198  
apple (*Malus*  $\times$  *domestica*) 241–282  
*Arabidopsis* 43  
automated temporary immersion system  
  (RITA) 481, 482, 486  
auxin 316, 322  
avocado (*Persea americana*) 167–187  
axillary buds 311, 390  
  
bacterial artificial chromosome (BAC)  
  399  
– library 293, 296, 337, 395, 504  
*Bacillus thuringiensis* 263, 339, 361, 456,  
  462, 488, 510, 542, 546  
– Bt toxin 456  
– Bt-engineered poplar 456  
– *cry1Ac* gene 488  
banana (*Musa* spp.) 3–5, 8–12, 20–23, 26  
– androgenic embryos 21  
– anther culture 21  
– breeding 4, 21  
– banana bunchy top virus (BBTV) 5  
– banana streak virus (BSV) 20  
– callus 12  
– Cavendish 12  
– climacteric 5  
– cucumber mosaic virus 20  
– ethylene 5–11, 25  
– explants 13  
– genes 5, 6  
– genetic variability 4

- genome 3, 5, 8, 9
- homozygous lines 21
- hybrids 4
- meristem cultures 12
- micropropagation 12
- microspores 21
- morphogenic responses 13
- mutation treatment 4
- production 3
- protoplasts 12, 13
- rhizomes 20
- shelf life 6
- shoot tip culture 12
- somaclonal variation 12
- suckers 12
- suspensions 12
- transgenic plants 21
- triploids 3
- vacuum infiltration 23
- benzylaminopurine 41
- biocontrol 264, 510
- biodiversity hotspots 498
- biofuel 458, 537, 538, 549
- biolistic (particle bombardment) 113, 316
- biomass 61, 105, 106, 257, 364, 379, 380, 387, 548
- bioreactor 20, 105, 110, 479, 481
- biotechnology 26, 58, 454, 460
- blackheart disease 118
- Botrytis cinerea* 318–320
- breeding 21, 26, 284, 285, 452, 461, 482, 483, 486, 489
- objectives 180, 330
- cacao (*Theobroma cacao*) 497
- caffeine biosynthesis 488
- Camellia sinensis* (tea) 519–535
- candidate genes 397
- Candidatus liberobacter asiaticum* 36
- carbohydrate metabolism 6, 7, 64, 256, 335
- carbon source 287, 416, 476, 481
- Casuarina glauca* 433–442
- Casuarinaceae 433–435, 437, 438
- cauliflower mosaic virus (CaMV) 35S 23
- CdSO<sub>4</sub> 11
- cecropin D 393
- cell wall 8–10, 63
- degradation 6, 316, 322
- proteins 8, 229, 438
- cellulase 171, 181, 322, 417, 419, 483, 484
- Cercospora coffeicola* 487
- certification 465
- cherry leaf roll virus 351, 360
- chimerism 22, 191, 315, 422
- chitinase 10, 25, 59, 65, 119, 181, 192, 194, 197, 200, 201, 266, 319, 320, 505, 509
- class III 10
- chocolate 497
- chromosome
- complement 98
- doubling 132, 151, 247
- counting 132, 133
- colchicine 132, 135, 136, 151, 222, 247, 340, 355, 485
- cinnamyl alcohol dehydrogenase (CAD) 392, 400
- Citrullus* 129
- *C. colocynthis* 130
- *C. ecirrhosus* 129
- *C. lanatus* 129
- *C. rehmii* 129
- citrus 3, 6, 11, 35
- Citrus mosaic virus* 46
- Citrus tristeza virus* 36
- climacteric 7, 8, 170
- clonal forestry 453, 462
- co-cultivation 23, 40
- cocoa 497–499, 501, 507, 510, 512
- coconut milk 103, 105, 107, 108, 478
- codling moth 361, 363
- codon usage 543, 546
- Coffea* 476
- Arabusta (*C. arabica* × *C. canephora*) 479
- C. arabica* 476, 478–481, 483–489
- C. arabica* × *C. canephora* 477
- C. canephora* 476, 479–481, 483, 485–489
- *C. liberica* 476, 485
- Congusta (*C. congensis* × *C. canephora*) 479
- coffee 475, 481–485, 487–489
- berry disease 487
- breeding 475
- dihaploids 484
- diseases 486
- embryogenic genotypes 478

- genetic transformation 486
- haploid plants 484
- homozygous plants 484
- interspecific hybridization 484
- leaf miner 488
- mass propagation 481
- mycorrhizas 486
- non-embryogenic (non-reactive) genotypes 478
- pests 486
- rust 483
- transformation 487
- Colletotrichum* 319, 320
  - *C. coffeanum* 487
  - *C. gloeosporioides* 59
  - *C. kahawae* 487
  - *C. kawaiae* 484
- color 332
- competent cells 40
- conifer 447, 448, 452, 456, 457, 466
  - biotechnology 447
  - economic importance 448
  - forestry 457
  - lignin-modified 457
  - plantations 448
  - wood supply 448
- consortium 396, 401
  - second Brazilian 396
- copper-chrome-arsenate (CCA) 458
- crown gall 350, 357, 362, 363
- cryopreservation 20, 57, 58, 181, 182, 191, 248, 338, 391, 507, 508, 512, 522
- Cryptomeria japonica* 464
- Cucumis* 209
  - *C. adana* 210
  - *C. ameri* 210
  - *C. anguria* 209
  - *C. cantalupensis* 210–212
  - *C. chandalak* 210
  - *C. chate* 210
  - *C. chinensis* 210
  - *C. conomon* 210, 212
  - *C. dudaim* 210, 212
  - *C. flexuosus* 210, 212
  - *C. hystrix* 209
  - *C. inodorus* 210–212
  - *C. makuwa* 210
  - *C. melo* (melons) 209
  - subsp. *agrestis* 210, 211
  - subsp. *melo* 210, 211
  - *C. metuliferus* 209
  - *C. momordica* 210
  - *C. reticulatus* 210, 212
  - *C. sativus* (cucumber) 131, 143, 149, 151–153, 156, 209, 210, 212, 213, 215, 222
- CuSO<sub>4</sub> 11
- cystatin 25
- cytochrome P450 6, 11, 199, 379
  - CYP71N1 11
- defensins 65
- determined proembryogenic cells (DPEC) 477
- dexamethasone 315
- dihaploids 484, 485
- disease 169, 170, 173, 176
  - apple scab 243, 254, 265, 267
  - black root rot 243
  - fire blight 243, 266
  - powdery mildew 243
  - resistance 65, 117, 211–213, 215, 225, 395, 544
  - *Rosellina* root rot 243
  - woolly apple aphid 242
- dissolved oxygen 481
- DKW medium 353, 354, 355, 356
- DNA fingerprinting 447, 454, 500
- double haploid 150, 217, 222
  - gynogenetic 151
  - inbreeding depression 151
  - irradiated pollen 151
- electroporation 22, 38, 250, 315, 487, 488, 538, 539, 543
- ellagic acid 309
- embryo 120, 285, 286
  - culture 285, 286
  - rescue 120
- embryogenic 20, 54, 177, 480
  - callus 20
  - capacity 480
  - cultures 54, 177
- endopolygalacturonase 317
- ESTs (expressed sequence tags) 6, 10, 11, 66, 153, 170, 192, 256, 258, 293, 296, 317, 335–337, 396, 397, 399, 401, 424, 504, 529, 530
- databases 335

- libraries 399
- resources 504
- ethylene 5-7, 11, 25, 220, 226, 227, 251, 252, 258, 259, 262, 267, 322, 480
  - autocatalytic inhibition 7
  - autocatalytic production 5
  - biosynthesis 6, 60
  - perception 227, 228
  - production 220, 223, 226, 227
  - polyphenol oxidase 253
- Eucalyptus* 387-406
  - *E. camaldulensis* 387, 389-393, 395
  - *E. dunnii* 396
  - *E. globulus* 387, 390, 391, 395, 396, 398, 400
  - *E. grandis* 387, 389-392, 395-398, 399, 402
  - *E. grandis* × *E. camaldulensis* 391
  - *E. grandis* × *E. globulus* 396, 399
  - *E. grandis* × *E. nitens* 396
  - *E. grandis* × *E. urophylla* 392, 398
  - *E. gunnii* 390, 391, 396, 397
  - *E. nitens* 390, 397, 398
  - *E. obligua* 396
  - *E. pellita* 396
  - *E. tereticornis* 390, 396
  - *E. urophylla* 390, 391, 396, 398, 399, 400
  - frost-tolerant 388
  - genetically modified 391
  - salt-tolerant 388
- expansin 8, 11
- explant 20
  - age 20
  - female flowers 20
  - immature male flowers 20
  - immature zygotic embryos 20
  - male flowers 20
- Fabaceae 407
  - Caesalpinioideae 407
  - Mimosoideae 407
  - Papilionoideae 407
- fatty acids 61, 360, 361, 510
- field collections 99
- field trial 46, 393
- flavor 9, 332
- flow cytometry 21, 38, 133, 135, 136, 139, 340, 391
- flower 9, 14-18, 20, 43, 47, 62, 63, 73, 81, 100, 111, 119, 131, 133, 134, 150, 167, 168, 194, 198, 199, 201, 212, 215, 227, 247, 254, 260, 294, 295, 318, 322, 330, 332, 333, 349, 351, 356, 360, 391, 407, 423, 424-426, 485, 506, 519, 520, 524
  - development 62, 424, 506, 507
- flowering 4, 36, 40, 43, 44, 53, 54, 74, 83, 99, 100, 102, 117, 119, 148, 149, 168, 179, 198, 199, 201, 241, 254, 336, 339, 358, 381, 396, 398, 407, 425, 520
- fluorocytosine 315
- forest 396, 449-451, 454, 460, 465
  - biotechnologies 451
  - certification 465
  - rotation 450
- forestry 427
- Fortunella* 35
- Fragaria* 309
  - *F.* × *ananassa* 314
  - *F. chiloensis* 309
  - *F. moschata* 309
  - *F. vesca* 309, 313, 314, 316
  - *F. virginiana* 309
  - *Fragaria* × *ananassa* 309
- fragrance 332
- Frankia* 434-439, 441
- frequency of outcrossing 66
- friable embryogenic tissue (FET) 479
- fructose 8
- fruit 5-11, 60, 98, 330
  - aroma 5, 10
  - climacteric 7
  - development 10, 11, 26
  - flavour 9, 10
  - fruit quality 60
  - overripe fruits 9
  - peel 7
  - post-climacteric 5, 7
  - pre-climacteric 5, 7
  - production 333
  - pulp 7
  - ripening 5-7, 60
  - ripening-related genes 6
  - softening 5, 8-10, 317, 322
- functional genomics 338
- fungi 65
- Fusarium oxysporum* 318
- Fusarium wilt 4

- GA<sub>3</sub> 416
- GC content 543
- GenBank 334
- gene-assisted selection (GAS) 447, 454
- gene
  - cloning 171
  - containment 424
  - diversity 66
  - flow 394, 422
  - manipulation 388
  - movement 462
- genetic engineering 21, 25, 140, 457, 458, 460, 461
  - *Agrobacterium tumefaciens* 140
  - banana 5
  - biolistic methods 142
  - tetraploid transgenic plants 143
- genetic
  - improvement 26, 36, 43, 63, 112, 119, 189, 192, 310, 352, 355, 388, 393, 439, 475, 484, 489, 499, 538, 541, 548
  - maps 66, 152, 156, 213, 216, 217, 256, 395, 398, 400
  - markers 37, 152, 156, 172, 215, 408, 423, 501, 512
  - modification (GM) 447, 464
  - relationships 99
  - variability 21
- genetic transformation 21, 84, 140, 180, 211, 218, 223, 225, 226, 229, 285, 291, 487–489, 508
  - *ipt* gene 292
  - abiotic stresses 229, 231
  - biolistic process 86
  - fruit quality 226, 228
  - method 223
  - particle bombardment 86
  - post-harvest 226, 227
  - transgenic plants 287, 291, 292
- Genolyptus project 395
- genome 26, 395
  - chloroplast 395
  - sequenced 395
  - small 395
- genomics 26, 156, 182, 190, 192, 255, 256, 267, 293, 294, 329, 330, 337–339, 361, 363, 388, 389, 394, 395, 399, 400–402, 451, 499, 502, 511, 512, 529
- germplasms 4, 5, 20, 40, 52, 57, 58, 82, 98–100, 102, 105, 111, 120, 131, 132, 155, 215, 225, 242, 248, 265–267, 283, 309, 310, 329, 330, 356, 357, 363, 391, 400, 419, 500, 501, 507, 508, 512, 525
- Gigaspora margarita* 486
- Glomus clarum* 486
- $\alpha$ -1,4-glucan-phosphorylase 8
- $\beta$ -glucuronidase (GUS) 41, 421
  - activity 41
  - expression 421
- Glucanases 6, 10, 25, 317, 419
- glucans 10
- glucose 11
- glufosinate 321
- glyphosate 321
- GM 455, 456, 460–466
  - trees 455
- grafting 37, 39, 40, 42, 51, 52, 81, 196, 242, 246, 248, 255, 286, 349, 350, 352, 353, 375, 512
- grape (*Vitis vinifera*) 189–208
- green fluorescent protein (GFP) 24, 41, 115, 118, 193, 194, 196, 297, 509
- growth 387, 392
  - regulators 411
- GTPases 66
- HAL1 gene 147
- haploids 3, 15, 21, 24, 37, 80, 132, 133, 135, 139, 150–153, 156, 168, 209, 211, 212, 216, 222, 242, 247, 337, 484, 485, 489, 508, 522, 528, 529
- Hemileia vastatrix* 487
- hepatitis B surface antigen (HBsAg) 26
- herbaceous legume 419
- herbicide
  - resistance 22, 222, 224, 358, 388, 393, 449, 457, 542
  - tolerance 114–116, 119, 120, 319, 321, 357, 439
- heterozygosity 36, 38, 66, 81, 172, 285, 296, 309, 408
- Hevea* 371–385
  - *H. brasiliensis* 371
- homeostasis 11, 148
- horizontal gene transfer (HGT) 462, 463
- HortResearch 329



- human
  - health 331, 460, 461
  - lysozyme gene 25
- hybrids 168
- hydrolases 63
- hyperhydricity 56, 174, 175, 178, 221, 245, 482, 486
- Hypothenemus hampei* 487
- in vitro 179, 218
  - callus 218–221
  - genetic control 221, 222
  - hyperhydricity 221
  - organogenesis 218, 220, 221
  - protocols 218
  - protoplasts 218, 221
  - somatic embryogenesis 218, 220
- insect pest 388, 393
- inter-fertility 394
- International Eucalyptus Genome Consortium 402
- interspecific hybrids 483
- introns 24, 217
- isomaltulose 548
- Juglans*
  - *J. hindsii* 350
  - *J. nigra* 351
  - *J. regia* 349
- juvenile growth phase 39, 173
- juvenility 39, 53, 62, 254, 354, 453
- kanamycin 38, 313–315
- late embryogenesis-abundant 321
- latex enzymes 87
- LEAFY* 43
- lectins 117, 320, 425, 542, 543
- libraries
  - subtractive 397
- lignin 354, 380, 388, 392, 393, 396, 397, 400, 422, 434, 439, 455, 457
- linkage maps 66, 152, 153, 262, 266, 293–296, 310, 340, 341, 360, 394, 398, 399, 423, 503, 527
- MaExp1* 11
- magainin 25, 145, 146, 194, 196, 197
- maize ubiquitin 25
- malate synthase (MS) 6, 9
- malic acid 9
- Malus* 241, 242, 248, 252, 254, 256
  - *M. baccata* 242
  - *M. floribunda* 242, 265
  - *M. micromalus* 242
  - *M. prunifolia* 242, 245
  - *M. robusta* 242
  - *M. sylvestris* 242
  - *M. x domestica* 241, 242, 254, 257, 265, 266
- Mangifera* 52, 57
- mango 51–71
  - anthracnose 51
  - cultivars 51
  - monoembryonic 47, 52, 54, 55, 57
  - seeds 58
- $\beta$ -mannanase 9
- MAP450-1* 11
- marker assisted
  - breeding 330, 388, 408, 423
  - selection 52, 152, 154, 173, 182, 213, 215, 255, 266, 294, 310, 323, 360, 388, 423, 501, 512, 525
- marker genes 40, 315
- marker-aided selection (MAS) 154, 447, 454
- markers 4, 5, 26, 152, 212, 215, 217, 244, 255, 266, 285, 293, 294, 454, 500, 525
  - amplified fragment length polymorphisms (AFLP) 4, 152, 153, 212, 293, 398
  - CAPS 525
  - EST 153, 294
  - fingerprinting 5
  - inter-simple sequence repeats (ISSR) 4, 153, 212, 525
  - microarrays 255
  - microsatellite DNA 4
  - QTL 266
  - random amplified polymorphic DNA (RAPD) 4, 21, 152, 153, 212, 246, 293, 398, 525
  - restriction fragment length polymorphisms (RFLP) 4, 212, 193, 296, 398, 525
  - sequence-tagged microsatellite site (STMS) 4
  - simple sequence repeat (SSR) 152, 153, 212, 213, 255, 256, 293, 294, 525

- single nucleotide polymorphism (SNP) 153, 212, 398, 399, 400
- variable number of tandem repeats (VNTR) polymorphisms 4
- mature phase 53, 174, 176
- meristems 12, 13, 14, 17, 20, 22, 24, 37, 62, 82, 102, 103, 110, 136, 177–179, 198, 199, 219, 246, 285, 297, 311, 354, 394, 408, 409, 401, 412, 422, 424, 485, 486, 541, 545
- cryopreservation 20
- germplasm preservation 20
- micropropagation 20
- metal 11, 262, 441
- detoxification 11
- metallothionein 199, 101, 441
- *cgMT1* 441
- metallothionein (MT)-like protein (MLP) 6, 10, 11, 530
- MT2A 11
- MT2B 11
- MT3 11
- methyl bromide 319
- 7-*N*-methylxanthine methyltransferase (CaMXMT1) 488
- microarrays 66, 255, 256, 317, 336, 505
- microcuttings 53, 83, 245, 390, 482, 485
- microfibril 317, 399
- micrografting 47, 175, 176, 181, 196, 246, 355, 523, 530
- micropropagation 81, 102, 173, 285, 311, 388, 390, 409, 485, 486
- acclimatization 82
- cloning 87
- rooting 81
- microsatellites 4, 37, 172, 173, 212, 256, 293, 361, 391, 398, 423, 527, 529
- microspores 21, 80, 484, 485
- model legume tree 426
- molecular mapping 502
- Musa* 3
  - *M. acuminata* 3, 6, 7, 19
  - *M. acuminata* ssp. *burmannicoides* 5
  - *M. balbisiana* 3, 19
  - *M. schizocarpa* 3
- mutation 58, 59, 89, 110, 111, 136, 179, 190, 218, 331, 340, 419, 461, 463, 502
- naphthaleneacetic acid 41
- nematodes 4, 25, 35, 98, 118, 295, 350, 363
- nitrogen-fixation 425
  - nodulation 436, 437
  - nodules 433–436, 438
- nitrogen-fixing 433
- nodule 442
- non-embryogenic 478
- nptII* 39
- nucellar 36, 37, 43, 54, 55, 57, 58, 62, 176, 248
- organic acids 9
- organogenesis 53, 219, 222, 247, 285, 291, 412
- ornamental pineapple 107
- oryzalin 135, 247
- Otiorynchus* 320
- $\beta$ -oxidation 61
- Panama wilt 25
  - *Fusarium oxysporum* f. sp. *cubense* 25
- papaya (*Carica papaya*) 73–94
  - diseases 74, 75
  - micropropagation 79
  - production 75
  - tissue culture 79
  - transformation 77
- papaya ringspot virus 75
  - coat protein 75
  - control 75
  - paper industry 387
- parthenocarp 149, 156, 193
  - gene DefH9-*iaaM* 149
  - PsEND1 promoter 149
- particle bombardment 21–23, 38, 86, 89, 102, 113, 114, 117, 118, 142, 287, 372, 374, 378, 419, 421, 488, 508
- patents 391
- pathogen resistance 388
- pathogenesis 5, 6, 10, 46
- pathogenesis-related (PR) proteins 10, 44, 59, 65, 146, 253, 425, 505, 506
- pectate lyase 6, 9, 317, 322
- pectin depolymerization 9
- pectin methylesterase 9
- peel 7
- Perileucoptera coffeella* 487
- Persea* 167
  - *P. americana* (avocado) 167
  - var. *americana* 167

- var. *drymifolia* 167
- var. *floccosa* 167
- var. *guatemalensis* 167
- var. *nubigena* 167
- var. *steyermarkii* 167
- var. *tolimanensis* 167
- var. *zentmyerii* 167
- *P. parviflora* 167
- *P. schiedeana* 167
- Persian walnut 349
- phenological characteristics 47
- Phytophthora* 36, 310
- *P. fragariae* 319
- pigments 61, 62, 201, 253, 260, 261, 371
- pineapple 97–127
- pinus (*Pinus* spp.) 450, 453
- *P. caribaea* 452
- *P. elliottii* 452
- *P. radiata* 452
- *P. taeda* 452
- *P. radiata* 456–458
- somatic embryogenesis 450
- plant
- architecture 61
- regeneration 21
- sterility 425
- plant–pathogen interactions 509
- plantain 22
- plantations 427, 448, 449, 456, 458, 460–462, 465
- forestry 456, 458, 460
- ploidy 219
- plum pox virus 296
- Podocarpus totara* 458
- pollen flow 360, 464
- polyembryonic 54
- polyethyleneglycol (PEG) 21, 38, 39, 338
- polygalacturonase (PG) 9, 63, 64, 66, 171, 172, 196, 228, 262, 317, 318, 335, 339, 340,
- polyphenol oxidases (PPO) 10, 11, 118, 253, 260, 261
- polyploid 133, 135, 136, 138, 142, 219, 247, 334
- Poncirus trifoliata* 35
- post-climacteric 7, 9, 10
- post-harvest losses 25
- Potyvirus* 75
- pre-climacteric 9–11
- preservation 82
- private companies 389
- production constraints 520
- promoters 23–25, 437, 439, 441, 442, 488, 538
- arabicin 488
- BBTv 24
- BSV 24
- cauliflower mosaic virus (CaMV) 35S 25, 440, 442, 488, 543
- *cg12* 437
- Emu 541
- Emu recombinant promoter 23, 451
- *enod12* 438
- *enod40* 436, 438
- maize ubiquitin 23, 24, 542
- Pubi-1(Ubi-1) 542
- RUBQ2 546
- sugarcane bacilliform badnavirus (ScBV) 23–24
- taro bacilliform badnavirus (TaBV) 24
- $\alpha$ -tubulin 488
- ubiquitin 24
- protease inhibitor 320, 544
- protoplasts 20–22, 38, 482–484
- yield 483
- culture 419
- density 20
- electrofusion 21
- embryogenic microcolonies 20
- feeder cells 21
- fusion 111, 483, 489
- isolation and culture 178
- nurse cell culture 21
- plant regeneration 20, 21
- polyethyleneglycol 21
- somatic hybrid 21
- source 21
- Prunus* 283–288, 291–297, 300
- *P. armeniaca* 283, 287, 296
- *P. avium* 283, 287, 296
- *P. canescens* 287, 296
- *P. cerasifera* 287
- *P. cerasus* 283, 287, 296
- *P. dawykensis* 296
- *P. domestica* 283, 287, 296
- *P. dulces* 296
- *P. dulcis* 283, 287
- *P. fruticosa* 287, 296
- *P. incisa* 296

- *P. persica* 283, 287, 296
- *P. pseudocerasus* 287, 296
- *P. salicina* 283
- *P. sargentii* 287
- *P. serotina* 287
- *P. serrula* 296
- *P. spinosa* 287
- *P. subhirtella* 296
- Pseudomonas syringae* pv. *garcae* 487
- pulp yield 9, 399
- pyramiding 244, 255, 265, 266, 462, 512
  
- quantitative trait loci (QTL) 154, 156, 213, 215, 266, 397, 399, 400, 454, 502, 503, 506, 538
  
- radiata pine 449, 454
- Radopholus similis* 25
- recalcitrant 389
- receptacle 316, 318
- reforestation 408
- regeneration problems 391
- resistance 25, 143
  - *Acidovorax avenae* 145
  - antimicrobial peptides (AMPs) 145
  - bacterial 143
  - cationic peroxidase 146
  - coat protein gene 143
  - fungal 143
  - movement protein-mediated 144
  - replicase-mediated resistance 144
  - systemic acquired resistance (SAR) 146
  - viral 143
- resistance gene analogs 506
- ribosome inactivating proteins 65
- ripening 7, 8, 10, 11, 26
- rolC* 42
- rootstocks 169, 242, 243, 245–248, 254–266
- Rosaceae 309
- Roundup 321
- rubber (*Hevea brasiliensis*) 371–386
  - *Agrobacterium*-mediated delivery 372, 373
  - anther callus 373
  - biolistic transformation 372, 374
  - biomass 379
  - bioreactor 376, 381
  - biosafety 381
  - biosynthesis 378
  - dryness 379
  - embryogenesis 372, 373, 379
  - feral rubber 381
  - genetic improvement 378
  - genetic transformation 372
  - latex 371, 374, 378
  - laticifer, latex vessels 374
  - molecular genetics 378
  - pollination 381
  - recombinant proteins 374
  - tapping panel 379
  - timber productivity 379
  - tissue culture 372, 373
  - transgene expression 376
  - xylogenesis 380
- Rutaceae 35
  
- Saccharum* spp. (sugarcane)
  - *S. barberi* 538
  - *S. officinarum* 538
  - *S. robustum* 538
  - *S. sinense* 538
  - *S. spontaneum* 538
- salicylic acid 481
- salt tolerance 147
  - Na<sup>+</sup>/H<sup>+</sup> antiporter, AtNHX1 147
- scion improvement 169
- screening 261, 266
- seed production 108
- seedling 401
  - realistic 401
- selectable markers 22, 292
  - *ipt* 292
- self-incompatibility 241, 254
- sex determinant 88
- sex-linked DNA 88
- sharka 294, 296
- shelf life 6, 25, 60, 75, 120, 211, 222, 250, 252, 259, 267, 310, 317, 322, 335
- shoot bud 413
- shoot tip
  - culture 20
  - grafting 37
- Sigatoka disease 12, 25
  - *Mycosphaerella musicola* 25
- resistance 4, 12, 24
- silver nitrate 133, 134, 480, 481
- social challenges 459

- somaclonal variant 110
- somaclonal variation 12, 120, 136, 285, 312, 482, 489, 547
  - adventitious shoot organogenesis 136
  - polyploid 136
- somatic embryogenesis 20, 109, 120, 177, 220, 285, 291, 475–478, 480–482, 488, 489, 507, 523
  - bioreactors 20, 105, 106, 110, 121, 246, 247, 364, 374, 376, 381, 459, 479, 481, 482, 523
  - high frequency SE (HFSE) 476
  - induction 478
  - in vivo embryogenesis 524
  - low frequency SE (LFSE) 476
  - secondary embryogenesis 523
- somatic embryos 20, 390, 477–484, 487
- somatic hybridisation 37, 111, 222
- somatic mutations 179
- sorbitol 252, 256, 258, 262, 267
- Sphaerotheca macularis* 319, 320
- starch 5, 7, 8, 63, 64, 99, 176, 256, 257, 259
  - amylopectin 7
  - amylose 7
  - degradation 7, 8
  - hydrolysis 8
  - synthesis 8, 176
- starch phosphorylase 7
- starch-debranching enzyme 7
- sterility 36, 42, 89, 150, 295, 333, 358, 394, 408, 422, 425
- storage 333
- strawberry (*Fragaria x ananassa*) 309–346
- stress-related genes 10
- stresses 6, 10, 392
  - abiotic 397
  - cold-acclimated 397
- sucrose 8, 11, 548
  - synthesis 8
- sucrose phosphate synthase 8
- sugar 5, 7, 537
  - synthesis 7
- sugarcane (*Saccharum* spp.) 537
  - herbicide resistance 542
  - insect resistance 542
- synthetic seeds 20, 245, 390
  - breeding objective 520
  - chromosomes 520
  - economic importance 519
  - originated 519
- tetraploids 52, 98, 131, 133, 135, 136, 138, 139, 142, 143, 209, 222, 242, 247, 334, 355, 485, 520
- texture 252, 255, 262
- thaumatin 64, 199, 200, 253, 261, 320, 331, 332
- Theobroma cacao* (cacao) 497
- therapeutic proteins 26
- thidiazuron 19, 220, 411
- timber 447–450, 455, 456, 458, 459, 465
- tissue browning 412
  - activated charcoal 412
  - antioxidants 412
  - ascorbic acid 412
  - citric acid 412
  - polyvinylpyrrolidone (PVP) 412
- tissue culture 52, 53, 79, 102, 337, 388, 521
  - anther culture 521
  - artificial seeds 521
  - callus 79
  - cryopreservation 521
  - embryo rescue 80
  - embryogenesis 79
  - micrografting 523
  - micropropagation 521
  - protoplast culture 521
  - protoplasts 80
  - regeneration 79
  - suspension 521
- transcription factors 229, 321, 340, 380, 392, 393, 397, 401, 425, 506
- transformation vectors 40
- transgene
  - dispersal 47
  - expression 23, 24
  - introns 24
- transgenic
  - conifers 456
  - plant 22, 23, 25, 26, 64, 296, 422, 487–489
  - trees 455, 464
- translational genomics 511
- tree 449, 450, 452, 454, 455, 461, 462, 464
  - breeding 450, 452
  - genetic engineering 454, 455
- tea (*Camellia sinensis*) 519–535

- herbicide resistance 457
- improvement 408, 452, 454
- insect resistance 456
- plantations 455
- tree legumes 407–431
- trifluralin 135
- triploid 3, 21, 37, 38, 98, 131, 132, 135,  
138, 212, 222, 242, 247, 337, 355, 356, 417,  
520, 526
- UDP-glucose 8
- UGPase 8
- uidA* 39
- vaccines 25
- vegetative propagation 399
- Verticillium dahliae* 319
- virus resistance 118
- virus-free plants 20, 311
- volatile esters 10
- walnuts (*Juglans regia*) 349–370
  - aflatoxin 362
  - allergen 364
  - early mature 360
  - fatty acid desaturases 360
  - functional genomics 361
  - genetic transformation 357
  - marker assisted selection 360
  - micropropagation 353
  - oil content 360
  - phytoremediation 364
  - shikimate dehydrogenase 362
  - somatic embryos 355, 364
  - walnut anthracnose 352
- watermelon 129–165
  - F1 hybrids 131
  - fruit characteristics 131
  - open-pollinated 131
  - seedless tetraploid 131
- wood
  - density 399
  - formation 397, 400
  - quality 387, 388, 392, 395, 398, 422
- zygotie embryos 177